THE BRITISH JOURNAL OF EXPERIMENTAL PATHOLOGY

VOL. XXII JUNE, 1941 No. 3

THE EFFECT OF CERTAIN ANTISEPTICS ON THE RESPIRATION OF BRAIN TISSUES *IN VITRO*.

M. C. MANIFOLD.

From the Department of Biochemistry, Oxford University.

Received for publication April 24, 1941.

THIS work was suggested to me by Prof. R. A. Peters, and originated from the purely practical aim of selecting an antiseptic which might be used with maximum safety in brain operations. It was carried out in parallel with work by Russell and Falconer (1940); the latter used histological methods, whereas here the relative toxicity to certain tissue enzyme systems was used as a means of comparison. Both these researches have led to similar conclusions, which gives confidence both in the conclusions reached and also in the value of the biochemical method of attack on a problem of this character. The majority of the experiments were made with the acridine series of compounds, though a few were made with other antiseptics. Briefly the work has shown that acriflavine is highly toxic and that proflavine sulphate and especially 2 : 7 diaminoacridine hydrochloride are much less so, though these latter compounds are known to be as efficient bacteriostatic agents as acriflavine.

Acriflavine.

Since Ehrlich and Benda (1913) introduced this substance into experimental medicine it has been widely used; it is a mixture of the hydrochlorides of 2 : 8 diaminoacridine and 2 : 8 diamino methyl-acridinium chloride and is highly bacteriostatic (Albert, Francis, Garrod and Linnell, 1938; Assinder, 1936; Browning, 1934; Davis, 1924; Hanschell, 1931).

A great divergence of opinion exists as to its effects upon the tissues. Certain authors have claimed that it is non-injurious in this respect (Hanschell, 1931; Suffolk, 1939; Young-Simpson, 1931), while others have testified otherwise (Russell and Falconer, 1940; Heathcote and Urquhart, 1930; Davis, 1924, 1932). Fleming (1940) even reported experiments in which he had demonstrated not only a lack of bacteriostatic action but a promotion of bacterial growth. This latter experience with acriflavine is not, however,

a general one and must be attributed to rather specialized experimental conditions.

There is, however, evidence of the clinical toxicity of acriflavine. Cullinnan (1931) observed acute toxic jaundice from the intravenous use of acriflavine in the treatment of gonorrhoea, and stated that there seemed to be good evidence that acriflavine given intravenously or orally 1/2 grain/day was capable of causing grave liver damage in man, confirming Murray (quoted by Heathcote and Urquhart, 1930) in this respect. Birch (1931) confirms these views, while Hanschell (1931) and Young-Simpson (1931) contradict them; the latter author states that "he had come to regard acriflavine as . . . near the ideal antiseptic as it was possible to obtain." Hughes and Birch (1933), in similar clinical work, claim that acriflavine caused local pain and abscesses at the site of the injection; retention of acriflavine in the body was noted, the plasma being for a long while fluorescent. Commenting upon the use of acriflavine in cases of gonorrhoea and *B. coli* infections of the urinary tract, Assinder (1936) revealed that between 1927 and 1933 the use of acriflavine intravenously had resulted in no serious clinical injury, but stated that between 1932 and 1933 jaundice began to occur with great frequency and the use of acriflavine was discontinued. In 1935 a preparation of acriflavine was marketed and found to be suitable for intravenous injection. This substance gave rise to only two cases of transient jaundice (48 hours) out of a total of 600. Further references to the injurious type of action were given by Davis (1924, 1932), who concluded that the indiscriminate use of acriflavine by the intravenous route was distinctly inadvisable. Walter (1932) investigated a number of compounds including acriflavine for antiseptic effects *in vivo*, and maintained that they were either so irritating to the mucosa of the gastro-intestinal tract, or so innocuous to bacteria as to be useless.

These adverse clinical effects are supported by experiments on the pharmacological and physiological affects of acriflavine. Meleney and Zau (1925) observed that acriflavine injected into rabbits (5–25 mg./kg.) invariably caused death within 6 to 25 days of administration, which was associated with degenerative changes in the liver and kidney. Heathcote and Urquhart (1930) noted a great fall in blood pressure on injecting 5–25 mg. acriflavine per kg. into dogs. Amounts of the order of 25–30 mg./kg. caused immediate death in most cases; this was not found to be due to paralysis of either the diaphragm muscles or the respiratory centre, but probably resulted from anaemia of the brain following a sudden and rapid fall of blood pressure. Crittenden (1932) also found that acriflavine in doses of 2–5 mg./kg. caused serious changes in heart rate, circulation and respiration when injected into dogs. Heathcote and Urquhart (1930) demonstrated that acriflavine concentrations as low as 1/1,000,000 depressed the isolated perfused frog heart, and that complete arrest in diastole followed the use of 1/10,000 acriflavine solution. This was mainly a direct action on the muscle and was not applied via the vagus. In contrast, Suffolk (1939) maintained that in the case of isolated frog's auricle, aqueous acriflavine of concentration 1/10,000 or less merely gave rise to staining, and that in a concentration of 1/100,000 the heart could survive as in optimal conditions from 2–3 days. An interesting effect of acriflayine was published by Heathcote and Urquhart (1930), who found

that a concentration of 1/10,000 caused complete depression of the rabbit's intestine, but those of 1/50,000–1/250,000 gave rise to a definite increase in tonus. These authors also showed that, applied at a concentration of 1/3000, acriflavine killed striated muscle but had little effect at concentrations between 1/20,000 and 1/50,000.

From the above it is clear that acriflavine itself may often be very toxic, and there is some evidence that this toxicity also extends to some of the biochemical components of the tissues, such as the enzymes. Dickens (1936*b*) observed that acriflavine had a marked inhibitory action on the Pasteur reaction of brain, and Quastel (1932) showed that at concentrations of 1/2000–1/3000 it would inhibit purified urease.

Other Acridine Derivatives.

Some work has already been recorded on the relative toxicity of acriflavine, proflavine sulphate and 2 : 7 diaminoacridine hydrochloride. Albert *et al.* (1938) have found the relative toxicities (L.D. 50 method) for mice to be 10–2·5–1. Suffolk (1939), using the isolated frog auricle, found the order for the inhibition of the acetylcholine effect to be 6–3–1, where 1 was cited as representing no inhibition.

EXPERIMENTAL METHODS.

The enzyme systems concerned with the oxidative degradation of carbohydrate (glycogen or glucose) must be considered to be of importance to the life of the cell, especially where (as in brain) the metabolism of the tissue is predominantly that of carbohydrate. It should be an ideal to obtain an antiseptic which, *inter alia*, satisfies the criterion of minimum toxicity to these enzymes. If it be thought that it is sufficient to find some compound which does not penetrate the cell membranes and so could not poison the tissue enzymes, it must be remembered that in exposed tissue surfaces, freshly cut or otherwise, it is unlikely that permeability relations are normal.

As the primary objectives here were emergency brain operations, brain tissue has been exclusively used, the tissue respiration being studied in oxygenated Ringer-phosphate solution with or without the addition of glucose or sodium pyruvate. CO_2-bicarbonate mixtures have not been thought necessary because (1) it has been clearly shown that the R.Q. (Dickens, 1936*a*) and the QO_2 (Dickens and Simer, 1930, 1931) give the same respective values whether they be measured in Ringer-phosphate or bicarbonate-Ringer media ; and (2) the main objects of the experiments were comparative. Sodium pyruvate was studied as well as glucose because of its interest as an intermediate in glucose degradation.

Brain tissue from either human, rabbit or pigeon sources, prepared as described below, was placed in suitable media in Dixon-Barcroft manometers, and the oxygen uptake with and without antiseptic was measured at a temperature of 38·2° C.

Three types of tissue preparation were used. These were slices 0·2–0·3 mm. in thickness, brei, i.e. well mashed tissue minus blood vessels and connective-tissue elements, and lastly finely ground dispersions. Details con-

cerning these different preparations are given by Gavrilescu, Meiklejohn, Passmore and Peters (1932) and Banga, Ochoa and Peters (1939). In the slice experiments each flask contained 50–80 mg. of wet tissue, the average number of slices per bottle being four. The amount of wet tissue used per bottle in brei experiments was 150–200 mg., while in dispersions about 350 mg. of tissue were employed.

The slice here represents the most highly organized preparation ; the dispersion has the structure practically destroyed. The Ringer-phosphate solution was used for slices and brei ; after grinding in the ice-cold mortar the dispersions were suspended in isotonic KCl to which phosphate buffer (pH 7·4) to M/20 and sodium fumarate to M/200 were added ; the latter is an essential component of the pyruvate oxidation system (Banga *et al.*, 1939). The antiseptics in neutralized form were added to the media immediately prior to incubation, the total volume of the media in the bottles being 3 ml. Experiments were made in duplicate, and values were not accepted unless they agreed to within 5 per cent. A full protocol of typical experiments is given in Table I.

TABLE I.—*Examples of Experimental Agreement. Effect of Different Concentrations of Acridine Antiseptics on the O_2 Uptake of Pigeon Brain Tissues* in vitro.

I. Dispersion with no substrate added.

	O_2 uptake µl/bottle/½ hr.			
	Exp. I.		Exp. II.	
Control . .	240	236	237	234
+2 : 7 1/500 .	94	90	85	92
+2 : 7 1/1000 .	179	184	185	191
+2 : 7 1/2000 .	189	186	189	181
+2 : 7 1/4000 .	199	201	194	197
+2 : 7 1/7500 .	199	192	202	206
+2 : 7 1/10,000 .	194	198	201	215

II. Slices in presence of sodium pyruvate.

	O_2 uptake µl/g./fresh tissue/hour.			
	Exp. I.		Exp. II.	
Control . .	2006	1972	1810	1760
+1/2000 profl. .	1024	1008	917	938
+1/4000 profl. .	1216	1144	1070	1140
+1/10,000 profl. .	1245	1261	1110	1086

2 : 7 = 2 : 7 diaminoacridine hydrochloride. Profl. = proflavine sulphate.

RESULTS.

The results shown in Tables I to IV and Figs. 1 to 9 are representative of some 60 experiments done under a number of different conditions, and conducted in such a manner as to be qualitatively and quantitatively comparable one with another. Figs. 1, 2 and 3 show a comparison of the effects of acriflavine, proflavine sulphate and 2 : 7 diaminoacridine hydrochloride on brain tissue in the form of slices, brei and dispersions. The results in Fig. 1 were obtained in the presence of glucose, while those of Figs. 2 and 3 were merely concerned with residual respiration. These three diagrams are examples from a large number of consistent experiments made with and without the addition of substrates such as glucose or sodium pyruvate, and serve to demonstrate the large differences in the toxic effects of the three antiseptics. The order of toxicity is not altered in the presence of added substrates. Fig. 4 shows that the inhibitory effect of acriflavine 1/1000 is exerted very markedly on

brain slices respiring in media with and without added substrates. A similar type of diagram holds for brei and dispersions, as has been found in a large series of experiments not reported here because they show no fresh facts. Such effects are exerted in all the types of brain tissue examined, and some

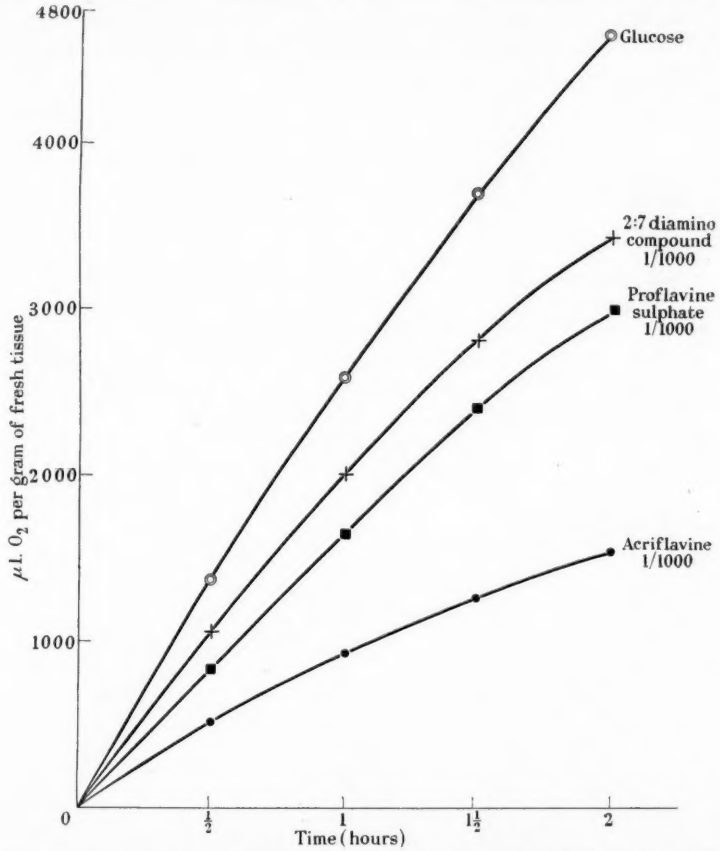

FIG. 1.—Brain slices (pigeon). Glucose. The effects of acriflavine, proflavine sulphate and 2 : 7 diaminoacridine hydrochloride at a concentration of 1/1000 on the O_2 uptake of pigeon brain slices in a medium containing glucose 0·02 M.; Ringer-phosphate pH 7·4, 38·2° C., oxygen gas, 2 hours' duration. Results of four experiments in each case.

results are presented in Table II to show the effects on different preparations from three animal types.

At a concentration of 1/1000 it is clear that 2 : 7 diaminoacridine hydrochloride is much less toxic than acriflavine, whether judged from the point of view of slices, brei or dispersions, and this compound has a remarkably low and uniform toxicity. Proflavine sulphate 1/1000 however is slightly more

toxic to slices than is 2 : 7 diaminoacridine hydrochloride, but its toxicity to dispersions approaches that of acriflavine. These results hold equally well for experiments made with or without the addition of substrates.

Whether the experiments were conducted using slices, brei or dispersions, the results are essentially the same except that (*a*) 2 : 7 diaminoacridine hydrochloride 1/1000 has little greater toxicity to dispersions than to slices (Figs. 1 and 3); and (*b*) for a given strength of any of the other antiseptics the

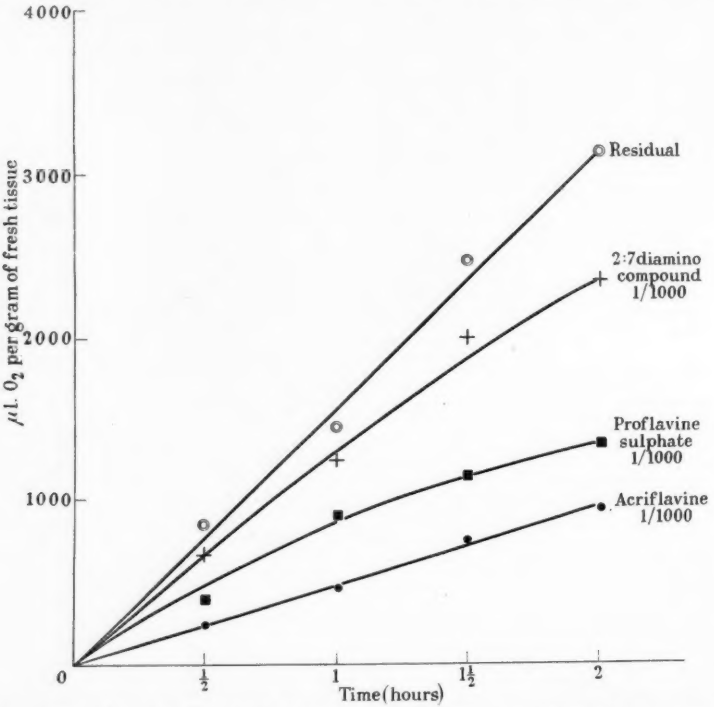

FIG. 2.—Brain "brei" (human). No additions. The effects of acriflavine, proflavine sulphate and 2 : 7 diaminoacridine hydrochloride at concentrations of 1/1000 on the respiration of human brain brei, in a medium devoid of added substrates. Ringer-phosphate pH 7·4, 38·2° C., oxygen gas, 2 hours' duration. Results of a single experiment.

toxic effects increase from slices through brei to dispersions. This is shown in Figs. 1, 2 and 3, and is provisionally interpreted to mean that 2 : 7 diaminoacridine as hydrochloride penetrates slices almost as well as dispersions, whereas the other two derivatives and particularly proflavine sulphate do not readily penetrate very deeply into the slices.

Two series of experiments using brain dispersions were performed to test the effects of acriflavine, proflavine sulphate and 2 : 7 diaminoacridine hydrochloride at different concentrations. Series I was concerned with residual

respiration only, while series II showed the effects in the total respiration (residual + pyruvate) in the presence of added sodium pyruvate. The results of these experiments are shown in Figs. 5, 6 and 8, and it is clear that the inhibitory effects of the antiseptics are greater in the presence of pyruvate than in its absence. The very high toxicity of acriflavine is made clear in Fig. 5, where the curves fall very gradually to a critical concentration and then steeply to a minimal value. With proflavine sulphate and 2 : 7 diamino-

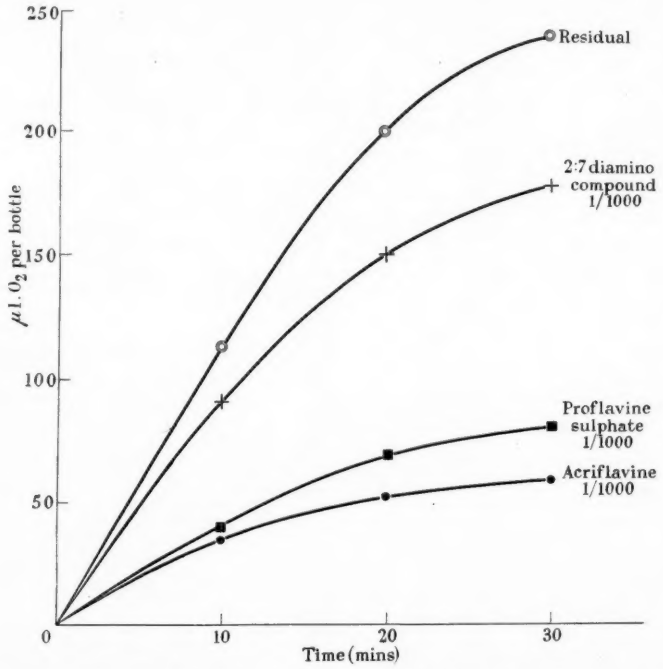

FIG. 3.—Brain dispersion (pigeon). No additions. The effects of acriflavine, proflavine sulphate and 2 : 7 diaminoacridine hydrochloride at concentrations of 1/1000 on the residual respiration of pigeon brain dispersions. Phosphate buffer M/20, pH 7·4, sodium fumarate M/200, 38·2° C., air, half hour duration. Results of three experiments in each case.

acridine hydrochloride however there is a rapid initial decrease in toxicity with dilution followed by a more gradual decrease to a minimal value.

In actual fact the toxicity of these compounds to the pyruvate oxidation enzyme systems is even greater than that expressed in these graphs. This was illustrated by means of an experiment in which the brain dispersion had been dialysed almost completely free from residual substrates. The respiration in this experiment was then due almost entirely to the oxidation of added pyruvate and the results of this experiment are shown in Table III.

TABLE II.—*The Effect of Acriflavine or Neutral Acriflavine* 1/1000 *on the Respiration of Brain Tissues from Various Sources.*

Ringer-phosphate pH 7·4, 38·2° C., O_2 gas, 2 hrs.

Experiment.	Duration.	Brain preparation and origin.	Added substrate.	Number of results.	O_2 uptake µl/g. of fresh tissue/hour. Antiseptic.		Percentage inhibition of the respiration due to acriflavine.
					Absent.	Present.	
1, 3, 6, 12, and 13	2 hrs.	Slices from pigeon hemispheres	Glucose	10	2360	708	70
5, 9, 14, 15, and 16	,,	Ditto	None	10	1084	459	59
2, 4 and 11	,,	Slices from rabbit cerebrum. Grey matter	Glucose	6	2063	653	69
7 and 10	,,	Slices from human cerebrum. Grey matter	,,	4	2470	474	80
8 and 20	,,	Brei from human cerebrum. Grey matter	None	2	1460	452	69
8 and 20	,,	Ditto	Glucose	4	2162	432	80
22	,,	,,	None	2	1460	452	69
17 and 18	1 hr.	Brei from pigeon hemispheres	,,	4	974	370	62
19	2 hrs.	Ditto	Glucose	2	2974	1220	59

TABLE III.—*The Effect of Proflavine Sulphate,* 2 : 7 *Diaminoacridine Hydrochloride and Acriflavine on the Respiration of Sodium Pyruvate* 0·016 *M. in Dialysed Pigeon Brain Dispersions.*

Phosphate buffer M/20, pH 7·4, 38·2° C., air, ½ hour duration.

Additions.	Total O_2 uptake µl/bottle/half hour.	Per cent. inhibition of the respiration due to the antiseptic.
None	402	—
Proflavine 1/1000	74	82
,, 1/2000	182	55
2 : 7 diamino. cpd. 1/1000	214	47
2 : 7 diamino. cpd. 1/2000	240	40
Acriflavine 1/100,000	96	76
,, 1/150,000	111	72

Curve C, Fig. 5, shows the effect of varying concentrations of acriflavine on the respiration of pigeon brain slices in a medium containing sodium pyruvate, and comparing this with curve A, Fig. 5, it is clear that for a given

concentration of acriflavine the effect on slices is much less than it is on dispersions. Fig. 7 shows the effect of different concentrations of proflavine sulphate on the respiration of slices in the presence and absence of glucose and sodium pyruvate. Compared with curve C, Fig. 5, curve B, Fig. 7 shows

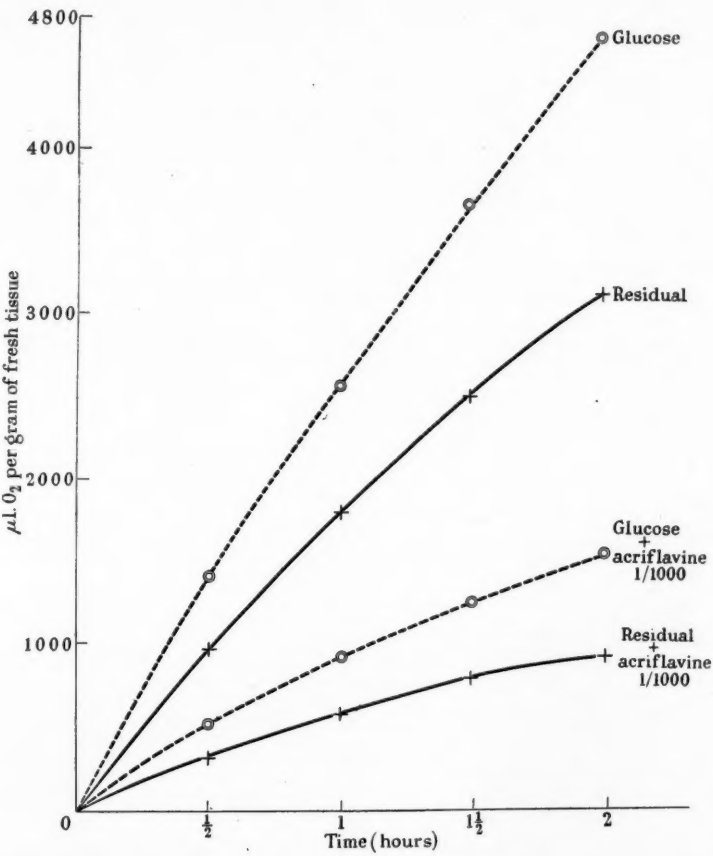

FIG. 4.—Brain slices (pigeon). With and without glucose. The effect of acriflavine 1/1000 on the respiration of pigeon brain slices in media with and without added glucose 0·02 M.; Ringer-phosphate pH 7·4, 38·2° C., oxygen gas, 2 hours' duration. Results of four experiments in each case.

a lower initial toxicity and a much more rapid fall to a minimal value, thus demonstrating the great difference between acriflavine and proflavine sulphate. Curves A and B, Fig. 7, illustrate the greater toxicity of proflavine to the total respiration of systems containing glucose or sodium pyruvate than to those

containing residual substrates only. It appears from these graphs that the toxicity for the glucose system is slightly greater than that for the one oxidizing sodium pyruvate, but this cannot be relied upon in the absence of more data. In order to obtain the most satisfactory comparison the data of previous experiments were considered, and concentrations of the three acridine compounds were selected which would give approximately 50 per cent. inhibition

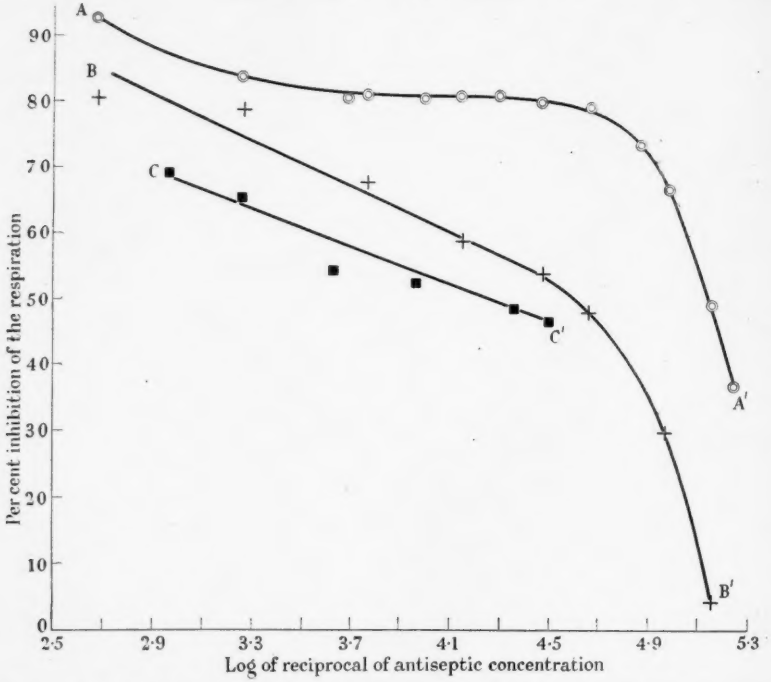

FIG. 5.—*Curves A and B.* Brain dispersion (pigeon). With and without sodium pyruvate. The effect of acriflavine on the respiration of pigeon brain dispersions in media with and without added sodium pyruvate 0·016 M.; phosphate buffer M/20, pH 7·4, sodium fumarate M/200, 38·2° C., air, half hour duration. *Curve C.*—Brain slices (pigeon). Sodium pyruvate. The effect of acriflavine on pigeon brain slices in a medium containing sodium pyruvate 0·016 M.; Ringer-phosphate pH 7·4, 38·2° C., oxygen gas, 2 hours' duration.

of the respiration. The results in Table IV show that acriflavine is fifteen times as toxic as proflavine and thirty times as toxic as 2 : 7 diaminoacridine hydrochloride as far as the residual substrates are concerned. When sodium pyruvate was added to the media, acriflavine was 100 times as toxic as proflavine and 300 times as toxic as 2 : 7 diaminoacridine hydrochloride.

Miscellaneous Antiseptics.

Two other antiseptics were tested by these respiration techniques. *Azochloramid*, used in the recommended concentration of 1/3300, was found to be

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Vol. 22, 121, 122. Illustrations only of Figs. 6 and 8 should be transposed.

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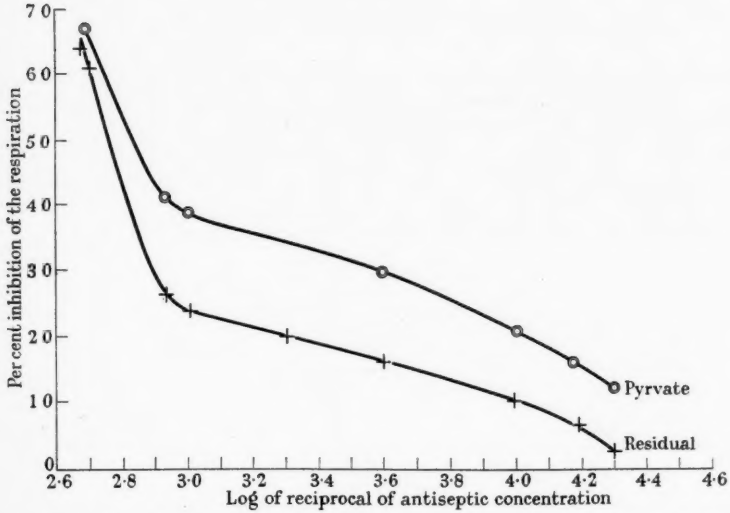

FIG. 6.—Brain dispersion (pigeon). With and without sodium pyruvate. The effect of proflavine sulphate on the respiration of pigeon brain dispersions with and without sodium pyruvate 0·016 m.; phosphate buffer M/20, pH 7·4, sodium fumarate M/200, 38·2° C., oxygen gas, half hour duration.

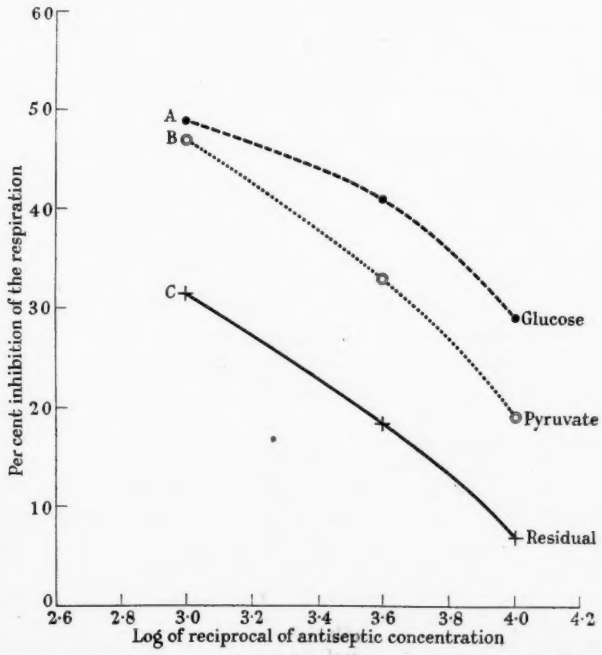

FIG. 7.—Brain slices (pigeon). With and without glucose or sodium pyruvate. The effect of proflavine sulphate on the respiration of pigeon brain slices in media with and without glucose or sodium pyruvate. Ringer-phosphate pH 7·4, 38·2° C., oxygen gas, 2 hours' duration.

extremely toxic to brain slices respiring in media with or without glucose. The percentage inhibition of the respiration with this concentration of the azochloramid was about 80, this figure being the average for a large number of experiments. On account of this high toxicity it was decided to determine what concentration of this substance would cause the minimal inhibition of the respiration. The average figure for this optimal concentration was 1/45,000, and the full results are expressed graphically in Fig. 9.

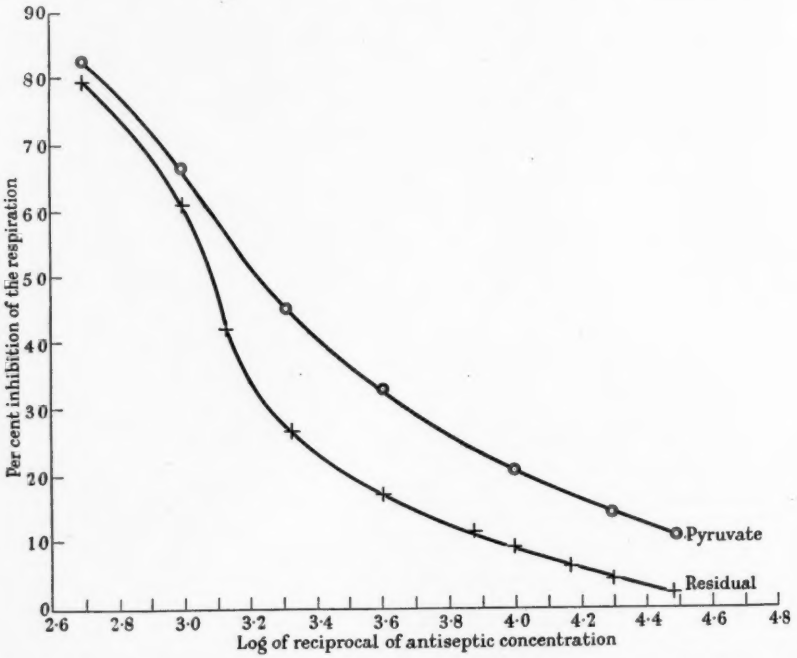

FIG. 8.—Brain dispersions (pigeon). With and without sodium pyruvate. The effect of 2 : 7 diaminoacridine hydrochloride on the respiration of pigeon brain dispersions with and without sodium pyruvate 0·016 M.; phosphate buffer M/20, pH 7·4, sodium fumarate M/200, 38·2° C., air, half hour duration. (Results of 3 to 4 experiments in each case.)

Experiments on soluseptasine, a sulphonamide derivative, showed that this compound caused a fairly high inhibition of the respiration of brain slices when it was used at the concentration recommended by the manufacturers, namely, 5 per cent. Fig. 9 shows the effect of increasing concentrations of soluseptasine on the respiration of brain slices placed in a medium containing 0·02 m. glucose. It is clear that there is no significant difference between the effect of 2·5 and 5 per cent. of this antiseptic, both of which concentrations cause a high inhibition of the respiration.

Experiments upon some antiseptics of the chlor-xylenol type, such as

dettol, were commenced, but these were discontinued because the antiseptics precipitated the respiration media, making work impossible.

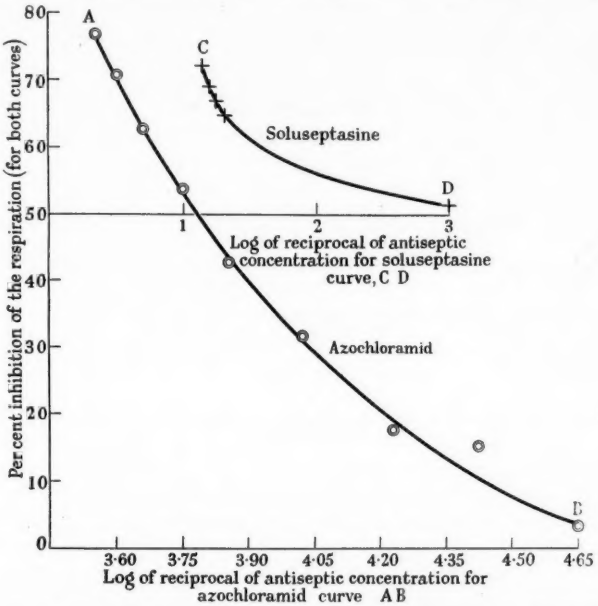

FIG. 9.—Brain slices (pigeon). Glucose. The effect of azochloramid on the respiration of pigeon brain slices in the presence of glucose 0·02 M.; Ringer-phosphate pH 7·4, 38·2° C., oxygen gas, 2 hours' duration. (Results of at least 3 experiments for each concentration of azochloramid.) Also, the effect of soluseptasine on the respiration of pigeon brain slices; media the same as for azochloramid experiments.

DISCUSSION.

The experimental data upon the brain enzyme systems support the work of those who have described a high clinical toxicity for acriflavine, confirm and amplify the biochemical data of Dickens (1936*a*, *b*) and Quastel (1932), and are in complete agreement with the pathological data of Russell and Falconer (1940) for rabbit brain.

The data of Albert *et al.* (1938) for mice, previously quoted, show a reasonably close agreement with the first column of the above table.

Suffolk (1939) used the isolated frog auricle to compare the acetylcholine inhibitory effect of acriflavine, proflavine sulphate and 2 : 7 diaminoacridine hydrochloride. The results were respectively 6 : 3 : 1, and show acriflavine to be relatively much less toxic than the results previously quoted. If the results of Suffolk be accepted they would indicate that acriflavine does not penetrate normal cells readily, or else that the acriflavine mixture of Suffolk was of a much less toxic character than that used by other workers.

So far I have not found any reliable method for discovering whether the

TABLE IV.—*Comparison of the Effect of Equi-toxic Concentrations of Acriflavine, Proflavine Sulphate and 2 : 7 Diaminoacridine Hydrochloride on Undialysed Pigeon Brain Dispersions With and Without Sodium Pyruvate, 0·106 M.*

Phosphate buffer M/20, pH 7·4, 38·2° C., air, 1/2 hr. duration.

Additions.	Residuals. μl O_2 1/2 hour per bottle.	Residuals. Per cent. inhibition of respiration.	Additions.	Pyruvates. μl O_2 1/2 hour per bottle.	Pyruvates. Per cent. inhibition of respiration.
None . . .	203	..	None . . .	498	..
Acriflavine 1/15,000	69	66	Acriflavine 1/150,000	150	70
Proflavine 1/1000 .	71	65	Proflavine 1/15,000 .	188	62
2 : 7 diamino compound 1/500	78	61	2 : 7 diamino compound 1/500	165	67

The relative toxicities of the acridine derivatives are as follows, the results being taken from Table IV.

	No substrate added.	Sodium pyruvate added.	Average toxicity ratio.
Acriflavine . .	30	300	165
Proflavine . .	2	3	2·5
2 : 7 diaminoacridine HCl .	1	1	1·0

action of these acridine antiseptics is reversible or not, and a similar type of difficulty was experienced by Quastel (1931). They appear to be strongly adsorbed on to tissue slices at least, and " reversibility " experiments using brain slices have led to no clear results. It seems that cells or enzymes which have been in contact with these derivatives do not recover their normality. The toxic effects of the compounds in wounds might be expected to be less severe, since in such cases penetration takes place in general from one surface only ; nevertheless the parallel experiments by histological methods of Russell and Falconer (1940) have shown that when applied to the living brain the order of toxicity is substantially the same as for the respiration experiments. It is obvious that *in vitro* experiments done with slices cannot imitate exactly conditions obtaining *in vivo*, but the general drift of the evidence seems clear.

It has been shown in a variety of ways that acriflavine as usually prepared is a substance of great toxicity to animal tissues, whether the toxicity be measured *in vivo* or *in vitro*. The toxicity seems to be related to a certain combination of groups in the molecule. Acriflavine is a mixture, and presumably may vary quite widely in composition from time to time and from one manufacturer to another. It is not significantly superior bacteriostatically to either proflavine sulphate or 2 : 7 diaminoacridine hydrochloride, both of which compounds, particularly the 2 : 7 diaminoacridine hydrochloride, have a minimal toxic effect on the tissues.

On these grounds it is felt that in spite of the wide interest in acriflavine its

use as a local or internal antiseptic should be discouraged, and proflavine sulphate or more particularly 2 : 7 diaminoacridine hydrochloride substituted, since these are pure compounds of invariable composition and low toxicity to tissues. The work of Albert *et al.* (1938), coupled with that of Russell and Falconer (1940) and that described in this paper, would lend the greatest support to the use of 2 : 7 diaminoacridine hydrochloride. This compound can now be prepared with comparative ease (Albert, 1940, private communication).

With regard to azochloramid and soluseptasine it was evident that neither of these was as satisfactory as proflavine sulphate or 2 : 7 diaminoacridine hydrochloride.

The essential similarity in the conclusions to be drawn from the results obtained by pathological methods upon living rabbit brain (Russell and Falconer, 1940) and by these simpler experiments *in vitro* upon certain brain enzymes needs emphasis. Although the enzyme method cannot properly test tissue permeability factors, it has the advantage of quantitative working and can be applied to enzymes from human brain. Further it appears within these limits to be valid; experiments so far have been confined to brain tissue.

SUMMARY.

Acriflavine, proflavine sulphate, 2 : 7 diaminoacridine hydrochloride, azochloramid and soluseptasine have been examined for their relative toxicity to glucose and pyruvate oxidation systems of brain tissue.

Acriflavine was found to be highly toxic even at very low concentrations. Proflavine sulphate was much less toxic than acriflavine, and 2 : 7 diaminoacridine hydrochloride was significantly less toxic than proflavine sulphate.

Azochloramid and soluseptasine were more toxic than proflavine sulphate at the concentrations recommended for their use.

The toxicities of the acridine derivatives for the enzyme systems examined agree closely with those obtained by histological and other methods. This supports the idea that the enzyme method can be used as a quantitative method for advancing knowledge of antiseptics, and indicates that it is safer to use proflavine sulphate or 2 : 7 diaminoacridine hydrochloride than acriflavine.

I wish to express my appreciation of the kind help and criticism afforded me by Prof. R. A. Peters, and to thank the Rockefeller Foundation for a grant in aid of this work.

I also wish to thank Dr. W. H. Linnell and Prof. Cairns who so kindly supplied me respectively with 2 : 7 diaminoacridine hydrochloride and human brain tissue.

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ANTI-LYMPHOCYTIC SERUM.

A. H. CRUICKSHANK.*

From the Sir William Dunn School of Pathology, University of Oxford, and the Department of Pathology, University of Aberdeen.

Received for publication April 7, 1941.

NUMEROUS workers have prepared antisera to the blood leucocytes. Sera effective against polymorphonuclear leucocytes have been obtained by Gladin (1901), Leschke (1913), Spät (1914), Ledingham and Bedson (1915), Bedson (1921 and 1922), Lindstrom (1927), Kimura (1928), Okita (1928), Yamamoto (1930), Witebsky and Komiya (1930), Heuper and Russell (1932), and Chew, Stephens and Lawrence (1936). Other workers, such as Metchnikoff (1899, 1900), Besredka (1900), Funck (1900), Flexner (1902), Bunting (1903), Christian and Leen (1905) and Pappenheimer (1917), used spleen or lymph node suspensions as antigen. The sera obtained by these workers seem to have had little specific effect upon lymphocytes and the majority of these sera were tested *in vitro*. Besredka found a leucocytosis in the blood of the animals which received his serum, but he did not carry out differential leucocyte counts. Flexner estimated the effect of his serum by the histological appearances of the lymphoid organs of the animals which received the serum. The effect was a hyperplasia of these organs. Bunting in his *in vivo* experiments, using lymphoid tissue as antigen, found that a lymphocytosis resulted when

* Medical Research Council Post-Graduate Fellow.

he compared the effect of his anti-serum with that of normal serum. Pappenheimer prepared a serum which he claimed was effective against lymphocytes and thymus cells when tested *in vitro* using the diffusion of trypan blue as an indication of cell damage. The only workers who obtained a satisfactory reduction of the circulating lymphocytes were Chew and Lawrence (1937). In the following experiments a method similar to that of Chew and Lawrence was used and similar results have been obtained.

MATERIALS AND METHODS.

The lymph nodes of young male albino rats were used as antigen. The rats weighed approximarely 150 g. each. To make the nodes easier to find the rats were injected subcutaneously with 1·5 c.c. of a 5 per cent. solution of the dye pontamine sky blue in normal saline (Sanders and Florey, 1940). The rats were killed with coal gas and the nodes were removed with sterile precautions ; the weight of nodes from each rat was about 0·4 g. Node tissue (0·2 g.) was triturated in a mortar with a little sterile sand, suspended in 3 c.c. of sterile saline and injected intraperitoneally into a rabbit. The injection was repeated every day for eight days and the rabbit was bled a week after the last injection. Six rabbits and one guinea-pig were immunized in this way and the serum obtained from all of them was effective against rat lymphocytes when tested *in vivo*. The sera from some of the animals were also tested *in vitro*.

After the animals had been bled the serum was separated from the clot and was heated to 56° C. for 30 minutes, cooled and mixed with washed packed rat erythrocytes. It was then incubated at 37° C. for 30 minutes and put into the refrigerator overnight. The erythrocytes from one rat were sufficient to absorb the haemolysins from the serum obtained from one rabbit. After being in contact with the rat erythrocytes overnight the serum was separated from the cells, again heated to 56° C. for 30 minutes and filtered through a Seitz filter and stored in sterile tubes in the refrigerator.

Blood counts.

The total white cell counts were carried out, using a Neubauer counting chamber. Blood was obtained by cutting the tip off the rats' tails under ether anaesthesia and was diluted 1 in 10 in Türck's fluid. On each occasion at least 500 cells were counted. The differential counts were carried out on smears stained by Leishman's stain ; 300 cells were counted, using the " cross-sectional " method (MacGregor, Richards and Loh, 1940), upon the body of the film. Red cell counts were also carried out from time to time during each experiment.

The large number of lymphocytes per cubic millimetre of blood in the normal rat made the rat a particularly suitable animal for these experiments.

RESULTS.

Effects of Serum in vivo.

Serum from rabbit No. 1 injected intraperitoneally into a rat in doses of 0·5 c.c. caused a fall in the circulating lymphocytes, which could be demon-

strated within an hour of the first injection (Fig. 1) and persisted for at least a week after the last injection of the serum. A control animal injected with similar doses of normal rabbit serum showed no such sustained fall in its circulating lymphocytes (Fig. 2). There was a slight increase in the numbers of polymorphonuclear leucocytes in both animals.

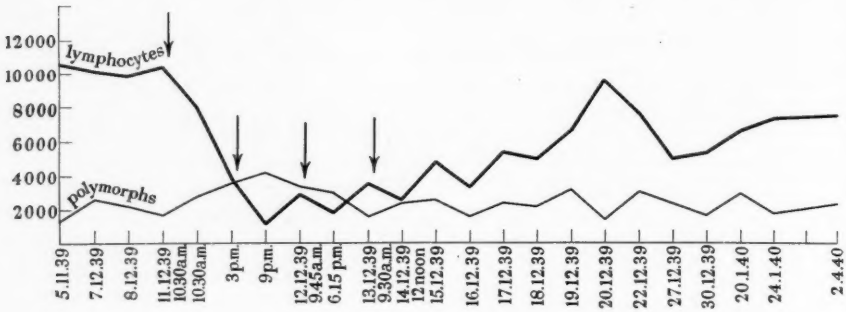

FIG. 1.—Antilymphocytic serum Rabbit 1. Doses of 0·5 c.c. indicated by arrows.

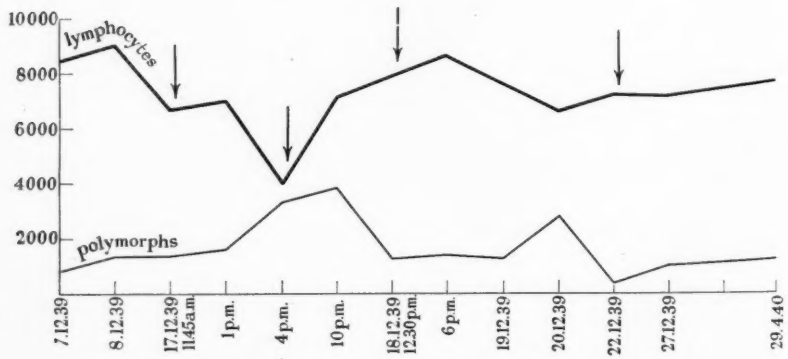

FIG. 2.—Normal rabbit serum. Doses of 0·5 c.c. indicated by arrows.

The effect of the anti-lymphocytic serum was confirmed by the experiments, the results of which are shown in Figs. 3 and 4. Although larger amounts of serum were used in these experiments the lymphocytes never completely disappeared from the circulation. Fig. 5 represents the lymphocyte and polymorphonuclear counts of an animal which received similar injections of normal rabbit serum.

In none of the animals was any significant change in the red cell count observed.

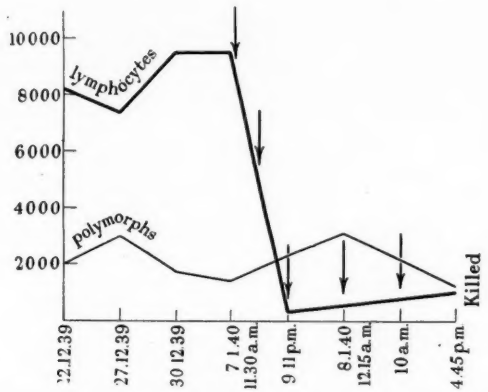

FIG. 3.—Antilymphocytic serum Rabbit 1. Doses of 1·0 c.c. indicated by arrows.

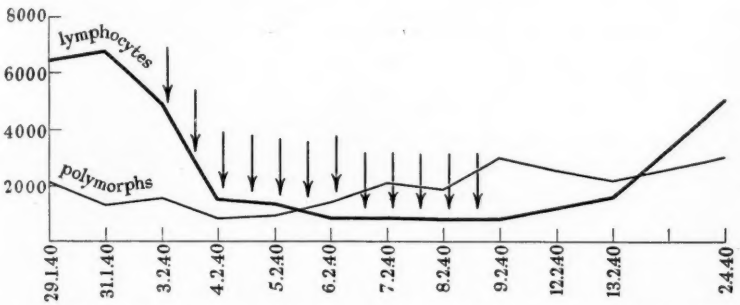

FIG. 4.—Antilymphocytic serum Rabbit 3. Doses indicated by arrows. The amounts given were 1·0 c.c. each for the first four and the last dose, and 1·5 c.c. each for the remainder.

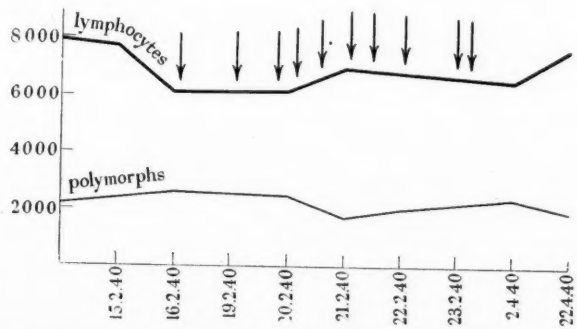

FIG. 5.—Normal rabbit serum. Doses indicated by arrows. The amounts given were 1·0 c.c. for the 2nd, 3rd and 4th doses and 1·5 c.c. for the remainder.

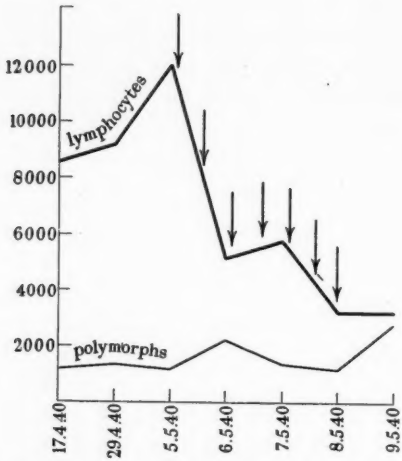

FIG. 6.—Normal rat. Doses of weak antilymphocytic serum indicated by arrows. The amounts given were 0·5 c.c. for the first three doses and 1·0 c.c. for the remainder.

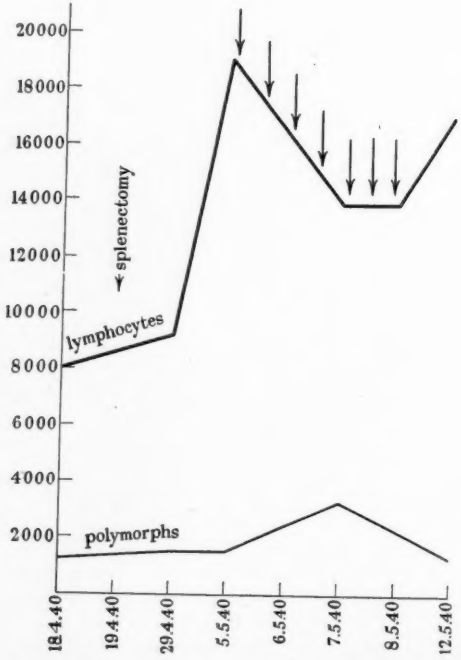

FIG. 7.—Splenectomized rat. Doses of weak antilymphocytic serum indicated by arrows. The amounts given were 0·5 c.c. for the first two doses and 1·0 c.c. for the remainder.

Effects of splenectomy upon the action of the serum.

Haemolytic immune sera were found to be less effective against red cells in splenectomized dogs (Pearce, Krumbhaar and Frazir, 1917) and this was

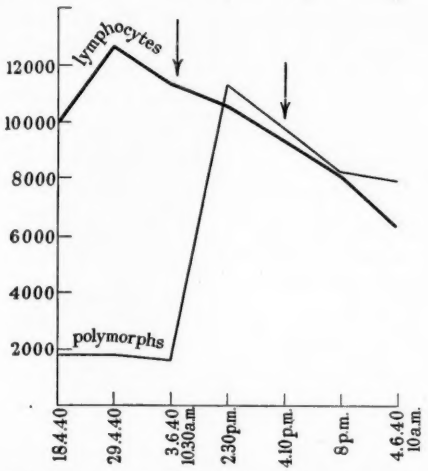

FIG. 8.—Normal rat. Guinea-pig antilymphocytic serum. First dose of 0·5 c.c. intravenous and second dose of 1·0 c.c. intraperitoneal indicated by arrows.

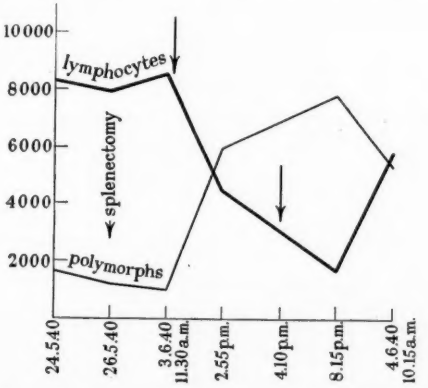

FIG. 9.—Splenectomized rat. Doses as in Fig. 8.

found to be true also in the case of splenectomized rabbits (Young, 1941). Experiments were carried out on rats in which the effects of the immune serum against lymphocytes were compared in normal and splenectomized animals. The results of these experiments are shown in Figs. 6 to 11. The serum which was used in the experiment shown in Figs. 6 and 7 was the weakest

serum obtained, and that used in the experiment shown in Figs. 8 and 9 was obtained from a guinea-pig. In the three experiments, however, there was little difference between the effects on the normal and on the splenectomized animals. This suggests that the spleen plays a smaller part in the removal of damaged lymphocytes from the circulation in the rat than it does in the removal of damaged and effete red cells in the dog and the rabbit. Before splenectomy the rats were injected subcutaneously with 3 mg. of neoarsphenamine and after splenectomy none of the animals showed signs of *Bartonella muris* infection.

In vitro *Experiments.*

A mixture of the serum from rabbits 5 and 6 was used in the *in vitro* experiments. This serum was used in the experiments shown in Figs. 10 and 11, which proved that it was effective *in vivo*.

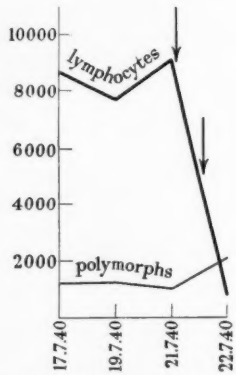

FIG. 10.—Normal rat. Mixture of antilymphocytic sera Rabbits 5 and 6. First dose of 1·5 c.c. and second dose of 1·0 c.c. indicated by arrows.

Agglutination.

The agglutination experiments were unsatisfactory. A suspension of lymphocytes was prepared by teasing lymph nodes in physiological saline and filtering the resulting suspension through gauze. The cells were then washed several times in saline. The number of cells in the suspension thus obtained varied greatly and the cells showed a natural tendency to form clumps. The number of cells in the suspensions varied on different occasions from 29,500 to 1400 cells per c.mm. This variation in the numbers of lymphocytes in the suspensions obtained by this method has been found by other workers (Turley and Dougherty, 1940). The effects of the serum to be tested also varied with different suspensions of lymphocytes, e.g. when one suspension of lymphocytes, containing 1720 cells per c.mm., was used definite slide agglutination was obtained, both macroscopic and microscopic, while the same

serum caused no agglutination of another suspension of lymphocytes, containing 1470 cells per c.mm. Normal rabbit serum caused no agglutination of these suspensions. When the suspension which had shown agglutination

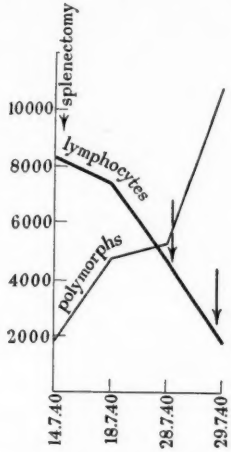

FIG. 11.—Splenectomized rat. Doses as in Fig. 10.

was tested with increasing dilutions of serum, agglutination was obtained in dilutions up to 1 in 40. Normal rabbit serum caused no agglutination in these dilutions.

Complement fixation.

In the following experiment the antigen was the same as that injected into the rabbits to produce the immune serum except that it was boiled for

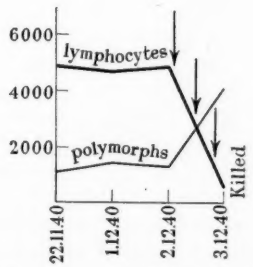

FIG. 12.—Rat. Three doses of 1·5 c.c. of antilymphocytic serum indicated by arrows.

10 minutes to reduce its anti-complementary activity. After boiling, it had to be triturated again to obtain a uniform suspension ; 1 c.c. of this suspension was not anti-complementary to one dose of complement (in this case the

minimum haemolytic dose of complement for 1 c.c. of sensitized sheep corpuscles was 0·02 c.c. of undiluted complement). The anti-lymphocytic immune serum had considerable anti-complementary activity and even 0·01 c.c. partially inhibited one dose of complement. With 0·02 c.c. of the serum one dose of complement was inhibited, but when three doses of complement were used haemolysis was complete.

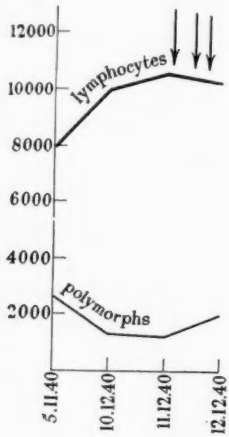

FIG. 13.—Mouse 1. Three doses of 0·5 c.c. of antilymphocytic serum indicated by arrows.

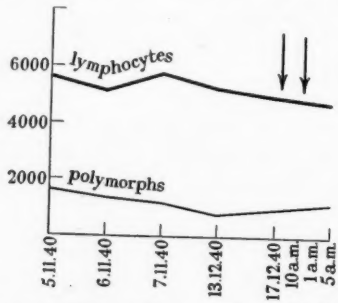

FIG. 14.—Rabbit 7. First dose of 5·0 c.c. and second dose of 3·0 c.c. of antilymphocytic serum indicated by arrows.

In the test 0·02 c.c. of serum was used and 1 c.c. of boiled antigen. These amounts were incubated with 3, 10 and 20 doses of complement for 1 hour, and then 1 c.c. of sensitized corpuscles was added and the tubes were incubated for a further hour. At the end of this period there was no haemolysis in the tubes containing 3 and 10 doses of complement, but haemolysis was complete in the tube containing 20 doses of complement. In a control series of tubes containing 0·02 c.c. of serum and 3, 10 and 20 doses of complement but no antigen, haemolysis was complete in all the tubes.

It was concluded that a true antigen-antibody reaction had taken place in which at least 7 doses of complement had been absorbed. Similar results were obtained with all the other batches of serum.

Species Specificity of the Anti-serum.

That the serum shows considerable specificity in its action against rat lymphocytes and not against the lymphocytes of other species is demonstrated in the following charts. Fig. 12 shows that the serum used in the experiment was effective against the lymphocytes of a rat weighing 140 g. It was given in three doses of 1·5 c.c. each intraperitoneally. Fig. 13 shows how little the lymphocytes of a mouse weighing 25 g. were effected when three doses of 0·5 c.c. each of the same serum were given intraperitoneally. The remainder of the batch of serum was given intraperitoneally in two doses of 5 c.c. and 3 c.c. respectively to a rabbit weighing 1·4 kg., and only an insignificant fall in its lymphocytes took place (Fig. 14).

HISTOLOGICAL OBSERVATIONS.

As was stated earlier, Flexner (1902), attempted to estimate the effect of his anti-serum by histological examination of the organs of the animals receiving it, and Pappenheimer (1917) found histological evidence of damage in the thymus of animals receiving anti-thymus cell serum, but he states that he was unable to repeat this experiment with uniform success. Chew, Stephens and Lawrence (1936) found a striking degree of myeloid hyperplasia in the bone marrow following injections of anti-polymorphonuclear immune serum and also a moderate degree of lymphoid and endothelial hyperplasia. After injections of anti-lymphocytic serum Chew and Lawrence (1937) could demonstrate a hyperplasia of the lymphoid tissue, but they found a similar hyperplasia in control animals receiving injections of normal serum. In the present work histological examination of the lymphoid organs was carried out in animals which were killed while their lymphocytes were at a low level, and the organs were compared with the organs of normal animals and animals which had received normal rabbit serum. The lymph nodes and spleens of the animals which had received injections of serum, either anti-lymphocytic serum or normal rabbit serum, showed proliferation of the sinus reticular cells, and in the case of the spleen this was sometimes well marked, but changes which could be attributed to any specific toxic effect of the anti-lymphocytic serum upon the lymphocytes of these organs could not be detected. There is evidence that there is normally a considerable " consumption " of lymphocytes as judged by the numbers entering the thoracic duct (the evidence is summarized by Sanders, Florey and Barnes, 1940). There is also evidence that, following excessive extirpation of lymph nodes, considerable hyperplasia of remaining lymphoid tissue occurs and new nodules are developed in the liver of rats (Sanders and Florey, 1940). It is therefore noteworthy that a low concentration of lymphocytes can be maintained in the blood following serum injections for as long as a week and little evidence of lymphocytic proliferation be evident in the nodes. The precise mechanism of the production of the low blood lymphocyte level following serum is not clear but, on the supposition

that they are destroyed in the blood stream, one would have expected to see some compensatory activity in the lymph nodes.

SUMMARY.

By the intraperitoneal injection of rabbits with a suspension of rat lymph nodes a serum is obtained which, after haemolysins have been absorbed, causes a marked reduction in the number of circulating lymphocytes in rats.

In vitro when the serum is placed in contact with a boiled suspension of rat lymph nodes an antigen-antibody reaction takes place in which complement is absorbed.

Unlike anti-erythrocyte immune serum the effect of the anti-serum upon the circulating lymphocytes is not modified by splenectomy in the animal which receives the serum.

No damage to the lymphocytes in the lymph nodes or spleens of the animals receiving the serum could be demonstrated.

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THE NATURE OF THE ANTIBACTERIAL AGENTS PRESENT IN *PSEUDOMONAS PYOCYANEA* CULTURES.

R. SCHOENTAL.

From the Sir William Dunn School of Pathology, Oxford.

Received for publication April 19, 1941.

THE study of antibacterial substances produced by micro-organisms which offers new possibilities of approach to problems of chemotherapy has recently received increased attention in various laboratories (Dubos, 1939 ; Chain *et al.*, 1940 ; Waksman and Woodruff, 1940*a*, 1940*b*).

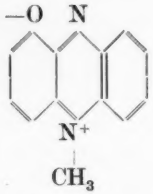
Pseudomonas pyocyanea (Ps.P.) is one of the oldest examples known of a micro-organism producing substances antagonistic to other bacteria (Bouchard, 1888). Emmerich and Loew (1899) and Emmerich, Loew and Korschun (1902) drew attention to the fact that old cultures of *Ps.P.* were highly bactericidal to many micro-organisms. They also noticed that such cultures concentrated ten times *in vacuo* had the interesting property of causing quick and complete lysis of thick suspensions of *V. cholerae* and *B. anthracis*. They ascribed this effect to the action of an enzyme, which they termed " Pyocyanase."

While the antibacterial properties of old *Ps.P.* cultures were confirmed, Emmerich and Loew's statement about its enzyme nature was contradicted by other workers (Klimoff, 1901 ; Dietrich, 1901), who considered the properties of the antibacterial preparations to be incompatible with those of an enzyme.

Subsequently, numerous papers on antibacterial preparations from *Ps.P.* appeared. Raubitschek and Russ (1908) showed that alcoholic extracts from *Ps.P.* cultures exhibited a considerable bactericidal action on *B. anthracis in vitro*, though some of the activity remained in the alcohol-insoluble fraction. They attributed the antibacterial action of old *Ps.P.* cultures to " lipoids." Fukuhara (1909) and Ohkubo (1910) confirmed the bactericidal, haemolytic and antitoxic action of alcoholic and acetonic extracts from *Ps.P.* and other bacteria. Hosoya (1929) claimed to have obtained a " lipoid " in crystalline form, and Hettche (1933, 1934) obtained from the bodies of *Ps.P.* a free acid, which he called " pyocyanic acid," of some bactericidal activity. Chatton (1927) found that saline extracts from *Ps.P.* agar cultures exerted a cytolytic action on different protozoa and Birch-Hirschfeld (1935) obtained similar bactericidal and cytolytic results with alcoholic extracts.

In addition to the " lipoid," another bactericidal substance has been known to be present in the *Ps.P.* cultures. The blue dye, pyocyanine, the pigment of " blue pus " (Fordos, 1860), the bactericidal action of which was already recognized and studied by Hettche (1932) and Kramer (1935), has been isolated

and synthesized by Wrede and Strack (1924, 1929). It was the first phenazonium compound discovered in nature, of the following structure (Hillemann, 1938):



On the suggestion of Prof. Florey and Dr. Chain the present work was undertaken to re-investigate the chemical nature and biological properties of antibacterial agents of *Ps.P.* cultures.

METHODS.

For the evaluation of the bactericidal action of *Ps.P.* cultures and their different fractions the manometric method (Bronfenbrenner, Hershey and Doubly, 1938, and Ely, 1939) was used, in which the rate of inhibition of bacterial respiration is followed. Two ml. of bacterial suspension in broth (16–24 hours old broth cultures or growth on agar slopes) were placed in the main compartment of a Warburg's vessel, and their oxygen uptake in air was measured at 37° C., in the usual manner. After 30 minutes' observation the substances to be tested, dissolved in 0·5 ml. of H_2O, were tipped in from the side bulb, and readings were continued for a further $1\frac{1}{2}$–2 hours. The results in Tables I, II and III give the actual oxygen uptakes during 30-minute periods, before and after mixing of the substance to be tested. The last column represents the percentage of inhibition of the oxygen uptake, during the second half hour after mixing.

Bacteriolysis was observed by removing the contents of the main compartment of the Warburg's vessel into small test-tubes and comparing the opacity of the suspensions with that of the controls, shaken for equal periods. The lysis of the bacteria seemed to be favoured by the mechanical shaking and mixing of the suspensions during the respiration experiments.

The growth inhibition of different bacteria was tested either in a fluid medium or on agar slopes. In the first instance, 5 ml. portions of 1 per cent. serum broth, containing the test substance in appropriate decreasing concentrations, were inoculated with one drop of an 18 hours old broth culture of the respective bacteria, using the standardized pipette of Dreyer (1920). The growth was observed during 24 hours at 37° C., by the macroscopical appearance of opacity. In the second case, 2 per cent. serum agar slopes, containing 0·1 per cent. of the test substance, were inoculated with loopfuls of the respective bacteria, and the growth compared with that of the controls, grown on 2 per cent. serum agar slopes at 37° C., during 24 hours.

For the growth of anaërobes heart broth, containing 20–30 mg. of l-ascorbic acid per 100 ml. of the medium was used. The use of l-ascorbic acid to produce a low oxidation-reduction potential was suggested by Prof. G. S. Wilson.

Anaërobiosis was maintained by a 1 cm. thick layer of cod liver oil. This medium could be autoclaved and kept for weeks, without change, and replaced the use of an anaërobic jar. A good growth of *Vibrion septique* in 24 hours could be obtained with an inoculum of 1 ml. of a 10^8 dilution of a 24-hours culture.

From toxicity tests on mice* it was clear that the substances from *Ps.P.* were too toxic to be injected for therapeutic purposes. Thereafter their action was tested on the growth of chicken embryo heart fibroblasts in tissue culture (Lambert, 1916 ; Salle and Lazarus, 1935 ; Salle, McOmie and Shechmeister, 1937). The coverslip technique was used, the different dilutions of the substance tested being made in embryo extract, and the growth of freshly explanted chicken embryo heart fibroblasts was observed during 48 hours.

RESULTS.

Preliminary Tests.

The antibacterial activity of different *Ps.P.* cultures was found to be associated with the following properties :

Dialysability.

Contrary to Emmerich and Loew's claim the greater part of the activity of *Ps.P.* cultures was found to dialyse through a " cellophane " membrane, during 48 hours, at 3° C.

Precipitability by alcohol.

Emmerich and Loew's claim (1902), that the active principle of *Ps.P.* cultures is precipitated by alcohol, could not be substantiated. Most of the activity was found to be extractable by alcohol, and only traces remained absorbed on the precipitate.

Heat resistance and resistance to the action of pepsin.

The resistance to boiling and to the action of proteolytic enzymes (Kulakowsky, 1909) was confirmed on all the fractions obtained by the above operations.

Extractability by organic solvents.

In view of the viscous nature of *Ps.P.* cultures, extraction of the active principles by organic solvents was impracticable in the fluid state. Good results were obtained after desiccation of the cultures by the lyophilic method. The fine powder thus obtained was extracted with choloroform, petrol ether or benzene in a Soxhlet apparatus. The starting material, as well as the extracted residue and the dried extracts, were dissolved in water, where necessary with the addition of alkali, neutralized, and their bactericidal action was tested by the manometric method on *V. cholerae*. The results are summarized in Table I.

It is evident that the substances responsible for all the antibacterial activity can always be quantitatively extracted by chloroform, and less effectively by

* I am indebted to Dr. J. M. Barnes for testing in mice the toxicity of different fractions and substances from *Ps.P.* cultures.

TABLE I.—*Inhibition of the Respiration of* V. cholerae *by a 3 months Old Broth* Culture of* Ps. pyocyanea *and its Fractions, Obtained by Extraction with Different Solvents.*

Sample.	Concentration mg./ml.	Solvent.	Yield. Per cent.	O_2 uptake in μl/30 mins. before and after mixing of the test substance.				Inhibition of O_2 uptake between 30–60 mins. Per cent.
				Before.	30 mins.	60 mins.	90 mins.	
Unextracted	10·0	..	..	78	8	6	4	−92
Extract	0·1	$CHCl_3$	13	77	12	0	0	−100
Residue	20·0	,,	13	89	89	130	135	+46
Residue	8·0	C_6H_6	4·5	120	76	100	101	−17
Residue	8·0	Petrol ether	2·0	162	66	45	42	−72
H_2O	..	..	..	123	159	195	240	+58

* The culture received six additions of 1% glucose.

a number of other solvents. Their yield and activity depend on the age and kind of cultures, as can be seen from Table II. Best results were obtained with heart broth cultures, to which 1 per cent. glycerine or glucose was added 10–14 days after the inoculation. This procedure of adding glycerine or glucose could be repeated several times at weekly intervals, fresh growth occurring after each addition.

The antibacterial substances could also be extracted from the bacterial bodies of *Ps.P.* in the same way. For this purpose bacteria were collected from 5 days old agar cultures, grown in Winchester quart bottles by the method of Epstein and Chain (1940), washed on the centrifuge and dried. They yielded 17 per cent. of the dried extract, possessing marked bactericidal activity (Table II). Another lot of 4 days old bacteria, washed on the centrifuge, were crushed for 4 hours in the bacterial mill of Booth and Green (1938). The bacterial residues were separated from the fluid by centrifuging for about $\frac{1}{2}$ hour at 15,000 r.p.m., washed and dried in a vacuum desiccator. They yielded on chloroform extraction 35·5 per cent. of an active dried extract, in the form of a slightly yellowish, semi-solid oil. The activity of ground bacteria was found mostly in the sediment, the supernatant being almost inactive.

Antibacterial Substances Isolated from Ps.P. *Cultures.*

The preliminary investigations of the crude extracts from *Ps.P.* cultures indicated that their antibacterial activity was due to, at least, three different substances :

1. Pyocyanine, the blue pigment, which appears in young cultures during the maximal growth of bacteria.
2. A yellow pigment appearing in older cultures, which was found to be a derivative of pyocyanine and identified with α-oxyphenazine.
3. An almost colourless, chloroform soluble, bacteriolytic substance.

Pyocyanine.

The isolation of this substance in crystalline form was performed by the method of Wrede and Strack (1924) from chloroform extracts of 48 hours old agar cultures containing 2 per cent. glycerine. The yield was approximately 60 mg. per Winchester quart bottle. Wrede and Strack, using broth cultures

TABLE II.—*Inhibition of the Respiration of* V. cholerae *by* Ps. pyocyanea *Dried Bacteria, Cultures and Different Fractions Obtained from Them by Extraction with Chloroform.*

Sample.	Concentration mg./ml.	Yield. Per cent.	O_2 uptake in μ/130 mins. before and after mixing of the test substance.				Inhibition of O_2 uptake between 30–60 mins. Per cent.
			Before.	30 mins.	60 mins.	90 mins.	
Bacterial bodies (5 days' growth):							
Extract	0·5	17	195	27	3	..	−98
H_2O	..	..	216	225	313	..	+45
Ground bacterial bodies:							
Unextracted	4·0	..	69	33	27	..	−61
Extract	0·5	35·5	84	28	28	..	−73
Extracted residue	20·0	..	51	88	105	..	+115
H_2O	..	..	81	84	75	..	−7
Broth culture (3 weeks old):							
Unextracted	4·0	..	117	72	79	56	−33
Extract	0·5	6	108	37	19	25	−82
Extract	0·2	..	110	78	85	97	−21
Extracted residue	20·0	..	96	165	170	171	+77
H_2O	..	..	123	159	195	240	+58
Broth culture (4 weeks old):							
Unextracted	5·0	..	135	61	21	13	−85
Extract	0·5	10	151	22	10	6	−93
Extracted residue	20·0	..	144	114	174	189	+21
H_2O	..	..	144	141	223	257	+39
Washings from agar c. (6 weeks):							
Unextracted	2·5	..	289	90	30	18	−90
Extract	0·2	13	243	45	14	1	−94
Extracted residue	10·0	..	263	252	252	248	+4
H_2O	..	..	256	271	235	213	+6
Broth culture + glycerin (3 months):							
Unextracted	10·0	..	64	12	7	4	−89
Extract	0·1	18	60	14	9	6	−83
Extracted residue	10·0	..	55	54	66	68	+20
H_2O	..	..	77	85	91	96	+18
Broth control:							
Extract	3·0	2·8	69	71	108	121	+58

TABLE III.—*Inhibition of the Respiration of* V. cholerae *and* Staphylococcus aureus *by Different Disinfectants.*

Substance.	Concentration. mg./ml.	O_2 uptake in μl/30 mins. before and after addition of substance. *V. cholerae.*					*Staphylococcus aureus.*				
		Before.	30 mins.	60 mins.	90 mins.	Inhibition. Per cent.	Before.	30 mins.	60 mins.	90 mins.	Inhibition. Per cent.
Pyocyanine	0·5	70	15	1	3	−99	74	21	9	4	−88
	0·1	65	30	22	19	−66	102	100	76	63	−25
	0·05	68	42	31	16	−54	..	..	..	..	..
	0·01	63	60	42	20	−33	..	..	..	..	..
	0	62	90	97	118	+57	..	..	..	..	..
α-oxyphenazine	1·0	241	46	7	3	−98	..	..	..	..	..
	0·5	..	..	..	..	..	76	22	10	6	−87
	0·1	279	46	45	12	−84	62	26	16	15	−74
	0·05	261	105	66	50	−75	..	..	..	..	..
	0	279	256	348	314	+25	79	111	159	183	+101
Crude III lytic factor	1·0	249	0	0	0	−100	82	21	15	9	−82
	0·5	213	18	2	1	−99	84	30	24	15	−71
	0·1	54	17	18	15	−67	78	48	64	72	−18
	0·05	54	22	20	29	−63	78	96	115	129	+47
	0	54	39	81	99	+50	89	145	248	252	+178
Purified III lytic factor	0·1	63	10	0	0	−100	..	..	..	..	..
	0·05	63	14	9	10	−86	..	..	..	..	..
Acriflavine	0·5	132	12	7	0	−95	..	..	..	..	..
	0·1	141	108	75	66	−47	177	107	66	48	−63
	0	120	130	177	189	+47	171	243	342	342	+100
Sodium lauryl sulphonate	10·0	70	9	7	7	−90	69	6	3	..	−95
	5·0	66	16	14	13	−74	66	5	9	..	−87
	0	75	55	60	57	−20	55	93	93	..	+69
Phenol	2·5	67	52	24	27	−64	..	..	..	..	..
Ichthyol	10·0	40	30	32	44	−20	48	0	6	3	−99
Sodium oleate	10·0	114	156	45	5	−60	..	..	..	..	..
Sodium olein.	2·0	..	..	..	..	..	293	172	117	89	−60
Sodium ricinoleate	2·0	..	..	..	..	..	375	108	79	69	−79

obtained only 1 mg. per litre. This result confirmed the observation of Gessard (1890), that glycerine present in the medium favoured the production of the dye by *Ps.P.* Its bactericidal action was tested on *V. cholerae* and *Staphylococcus aureus* by the manometric method, and the results are given in Table III.

The strong bactericidal activity of pyocyanine is accompanied by a high toxicity for white mice. The M.L.D. with intraperitoneal injections was about 2 mg. Its action on the growth of fibroblasts is shown in Table VI.

α-oxyphenazine.

Ps.P. cultures left in the incubator longer than for 8–10 days turn brownish-yellow. From such 2–4 weeks old glycerine agar cultures, a dark brown chloroform extract was obtained. Passing it through a column of aluminium oxide, standardized according to Brockmann, a yellow substance was separated. This was easily eluted by dilute alkali, while other components remained adsorbed on the first layer of the column. After acidification of the red-violet eluate a yellow flocculent precipitate was formed, which could be purified either by reprecipitation and extraction with chloroform, or by sublimation at a reduced pressure. The purified product presented yellow prismatic needles, readily soluble in alkali, producing a deep red-violet solution.

The same substance was obtained from an alkaline solution of pyocyanine on standing in air, which gradually turned from blue to red-violet. This yielded on acidification and extraction as above yellow crystals, identical with those obtained by the above method from crude extracts. The colour reactions of this substance (yellow at pH 2–9; red-violet above pH 10; and pink below pH 2), its phenolic character, solubility in alkali, crystalline form, sublimation and melting point, not depressed by admixing of pure α-oxyphenazine,* suggest its identity with α-oxyphenazine, as synthesized first by Wrede and Strack (1928).

α-oxyphenazine exhibited a strong bactericidal action towards *V. cholerae* and *Staphylococcus aureus*, when tested by the manometric method, comparable to that of acriflavine and pyocyanine (Table III). It inhibited the growth of many bacterial strains, including anaërobes, at a concentration of 1 : 1000 (Table IV). Only a few strains, such as *B. dysenteriae*, *Bact. flexneri* V, *Bact. paratyphosum* A, etc., resisted its action under these conditions.

The growth-inhibiting action of α-oxyphenazine was compared with that of proflavine on some bacteria in a fluid medium. The results are shown in Table V. The apparently weaker action of α-oxyphenazine may be due to its gradual inactivation under these conditions. A colour change to a deeper brown was observed after incubation with bacteria at 37° C. for 24 hours. During the first 6–8 hours after inoculation the growth of *V. cholerae*, for instance, was inhibited down to a concentration of 1 : 750,000.

The toxicity of α-oxyphenazine is relatively low. Owing to its low solubility a suspension of 10 mgm. was injected intraperitoneally; it was not lethal for mice. The growth of chicken heart fibroblasts was not inhibited in the presence of 0·2 mg./ml. (Table VI).

* The sample of α-oxyphenazine was kindly supplied by Dr. H. McIlwain, to whom I wish to express my thanks.

TABLE IV.—*Growth Inhibition of Different Bacteria by α-oxyphenazine.*

Strain of bacteria.	Growth during 24 hours at 37° C.	
	Control.	0·1 per cent. α-oxyphenazine.
C. diphtheriae gravis	+++	—
B. anthracis	+++	—
Neisseria meningitidis	+++	—
V. cholerae	+++	—
V. metchnikovi	+++	—
V. El-Tor	+++	—
Strep. pneumoiae (Type 1)	+++	—
Strep. haemolyticus	+++	—
Strep. faecalis	+++	± ?
Staphylococcus aureus	+++	—
Staphylococcus, bovine	+++	—
Bact. shigae	+++	—
Bact. sonnei	+++	—
Bact. flexneri V	+++	++
Bact. typhosum H	+++	—
Bact. typhosum O	+++	—
Bact. paratyphosum A	+++	++
Bact. paratyphosum B	+++	+
Bact. coli	+++	+ ?
B. subtilis	+++	—
Proteus vulgaris	+++	—
Chr. prodigiosum	+++	—
Br. melitensis	+++	—
B. megatherum	+++	—
Br. abortus (6 days)	+++	—
Bact. friedländeri	+++	+
Cl. welchii	+++	—
Cl. septique	+++	—
Cl. tetani	+++	—
Sarcina lutea	+++	+

TABLE V.—*The Concentration of Some Antibacterial Substances, Inhibiting the Growth of Bacteria, in Serum Broth during 24 Hours at 37° C.*

Substance.	*Staphylococcus*, bovine.	*Streptococcus haemolyticus.*	*Pneumococcus.*	*V. cholerae.*	*B. aertrycke.*
α-oxyphenazine	1/20,000	1/20,000	1/20,000	1/40,000	1/2000
Proflavine	1/50,000	1/100,000	1/4000	1/100,000	1/10,000
Purified III.	1/20,000	1/2000	1/5000	1/100,000	..

The bacteriolytic factor.

It was noticed that in 2–3 months old broth cultures α-oxyphenazine underwent further changes, forming a black-brown, amorphous substance, no longer bactericidal and insoluble in chloroform and other solvents. On

TABLE VI.—*Effect of Antibacterial Substances on the Growth of Fibroblasts in Tissue Culture.*

Substance.	Concentration mg./ml. 5·0.	1·0.	0·5.	0·2.	0·1.	0·075.	0·05.	0·025.	0·000.
Pyocyanine	..	−	−	−	±	+	++	+++	+++
α-oxyphenazine	..	−	−	++	+++	+++	+++	+++	+++
Bacteriolytic substance III	−	±	+	+++	+++	+++	+++	+++	+++
Acriflavine	..	..	−	−	−	−	±	++	+++
Proflavine	..	..	−	−	−	−	++	−++	+++
Sulphanilamide	+++*	+++	+++	+++	+++	+++	+++	+++	+++

... = not tested.
− = no growth.
+ = few cells surrounding the explant (migrating ?).
+++ = growth similar to the control.

* The insolubility of sulphanilamide in higher concentrations prevented its further testing. It is clear that sulphanilamide does not inhibit the growth of fibroblasts, even in a medium saturated with it. Hence, it should not interfere with the healing of wounds when applied in crystalline form.

extraction of such dried broth cultures with chloroform a pale yellow oil was obtained, containing only small amounts of acetone-insoluble substances. It possessed acid properties, being soluble in dilute alkali and forming insoluble salts with calcium, barium and heavy metals. The ether-soluble lead salt was used for its purification. The purified active material is an almost colourless oil, possessing a characteristic, not disagreeable smell.

This product exerts a strong bactericidal action towards *V. cholerae* and *Staphylococcus aureus*, as tested by the manometric method (Table III). Its action on *V. cholerae*, *V. metchnikovi*, *V. El-Tor*, etc., is characterized by lysis of these organisms accompanied by the formation of a peculiar gel. Thus, a thick broth suspension of *V. cholerae*, etc., is lysed down to the concentration 1 : 10,000, while growth inhibition of *V. cholerae* may be noted even in as low a concentration as 1 : 100,000, though its bacteriostatic action towards other bacteria is much smaller.

Its toxicity is not very great. The M.L.D. is about 5 mg. per mouse when injected intravenously, though this amount is not lethal when injected by other routes. The growth of chicken fibroblasts is not inhibited in the presence of 0·3 mg./ml.

DISCUSSION.

The above results indicate that the antibacterial activity of *Ps.P.* cultures is not affected by boiling, or the action of pepsin ; moreover it was found that this activity is due to substances which are dialysable, soluble in alcohol, and which can be quantitatively extracted by chloroform, from cultures and bacteria dried by the lyophilic method. Hence, they are not of an enzymatic nature. From such chloroform extracts three bactericidal substances were obtained : Pyocyanine, the blue pigment, isolated from extracts of 2–3 days old cultures in crystalline form ; α-oxyphenazine, the yellow pigment of older cultures, isolated in crystalline form ; and an almost colourless, oily substance

obtained in extracts from 2–3 months old cultures, and purified by its ether-soluble lead salt.

In Table III are summarized the results of the action of these individual substances on the respiration of *V. cholerae* and *Staphylococcus aureus*, and compared with that of other disinfectants, such as flavine, phenol and salts of some unsaturated fatty acids, while Table VI shows the action of some of these substances on the growth of fibroblasts in tissue culture. Pyocyanine, in spite of its strong bactericidal activity, does not appear to be promising for therapeutic purposes, owing to its high toxicity and instability. The antibacterial properties of α-oxyphenazine, which may be identical with the " Pyoxanthose " of Fordos (1862) and has since been synthesized by Wrede and Strack (1928), have till now not been recognized. It exhibits strong antibacterial action towards many bacterial strains, comparable to that of the flavines, it is less toxic, it is not irritating and it possesses no marked staining properties.

In their extensive studies of the antibacterial properties of compounds of the acridine and phenazine groups, Browning, Cohen and Gulbransen (1920) and Browning, Cohen, Gaunt and Gulbransen (1922) have investigated a great number of amino-, and methylamino-phenazonium compounds. The presence of hydroxy-phenazonium compounds in *Ps.P.* cultures, of a high antibacterial activity, merits further investigation.

The active constituent of the almost colourless material extracted by chloroform from old *Ps.P.* cultures is possibly related to Hettche's " pyocyanic acid." Its antibacterial activities, in the crude and purified form, are summarized in Tables III and V. It exhibits a bactericidal action on *V. cholerae* down to a concentration 1 : 10,000, and a bacteriostatic action down to a concentration 1 : 100,000, comparable to that of proflavine, tested under the same conditions. Its action towards other bacteria is much weaker. Its most striking feature is its lytic action on vibrios, down to a concentration 1 : 10,000. Of all the substances tested only sodium lauryl-sulphonate exhibited analogous lytic action, and that only in higher concentrations. The bacteriolytic action of sodium lauryl-sulphonate has been already studied by Bayliss (1937), and its bacteriostatic activity by Birkeland and Steinhaus (1939). The antibacterial action of salts of unsaturated fatty acids is much weaker.

SUMMARY.

The substances responsible for the antibacterial activity of *Ps. pyocyanea* cultures are heat stable, resistant to the action of pepsin, soluble in alcohol, and can be extracted quantitatively by chloroform from cultures dried by the lyophilic method. Hence they have not an enzymatic nature.

Three antibacterial substances were isolated from the chloroform extracts of *Ps. pyocyanea* cultures : (1) pyocyanine, (2) α-oxyphenazine, and (3) an almost colourless bacteriolytic substance.

The antibacterial activities of these substances were compared with those of other disinfectants, and their action on the growth of fibroblasts in tissue culture were studied.

The main feature of the third antibacterial substance from old *Ps. pyocyanea* cultures is its strong action on *V. cholerae*, accompanied by a lysis of the vibrios and gel formation.

I am greatly indebted to Prof. H. W. Florey for his kind help and encouragement, and hospitality in his Department. My thanks are due to Dr. E. P. Abraham and Dr. I. Berenblum for their kind correction of the manuscript, and to Mr. A. L. Haynes for the preparation of different bacterial media.

I wish to thank the Nuffield Trust for a personal grant, which enabled this research to be carried out.

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BACTERIAL INHIBITION BY AMINOSULPHONIC ANALOGUES OF SOME NATURAL AMINOCARBOXYLIC ACIDS.

HENRY McILWAIN.*

From the Department of Bacterial Chemistry (Medical Research Council), Bland Sutton Institute of Pathology, and from the Courtauld Institute of Biochemistry, Middlesex Hospital, London, W. 1.

Received for publication May 5, 1941.

BACTERIAL growth, inhibited by the addition of certain sulphonic acids or their amides to culture media, can be restored by the further addition of the corresponding carboxylic acids or by derivatives of them (Woods, 1940; McIlwain, 1940*b*). It is considered that the carboxylic compounds take part in reactions, essential to growth, which are retarded or prevented by blocking at enzyme surfaces by the similarly constituted sulphonic derivatives (Fildes, 1940). Many aminocarboxylic acids are known to be involved in growth of micro-organisms, often constituting limiting factors in growth of pathogens. Sulphonic acids corresponding to several of these—to glycine, alanine, valine, leucine, β-alanine and aspartic acid—have accordingly been examined as bacterial inhibitors.

1. TECHNICAL DETAILS.

Aminosulphonic compounds were those prepared by McIlwain (1941) with the addition of cysteic acid and a commercial specimen of taurine. Neutral $M/10$ solutions were sterilized by filtration.

Organisms and conditions of growth.—Laboratory strains of *Bact. coli*, *Staphylococcus aureus* (strain I of Gladstone, 1937*b*), *Proteus vulgaris* and other strains of the latter organisms from the National Collection were grown as previously described (McIlwain, 1940*b*), nicotinic acid being added where necessary. *Proteus morgani* was grown as the *vulgaris* strain with the further addition of pantothenate ($10^{-7}M$; Pelczar and Porter, 1940). The normal *Bact. typhosum* was a strain from Dr. A. Felix, grown in a medium based on the amino acid medium of Gladstone (1937*a*) containing lactate, but containing in addition aneurin ($10^{-7}M$) and pantothenate concentrates (McIlwain, 1940*a*). The trained organism was grown in the ammonia medium of Gladstone (1937*a*) and the trained staphylococcus in the ammonia-cystine medium (Gladstone, 1937*b*), using in all these cases, however, small inocula of the same size as with normal strains (1000 or 10,000 organisms). *Corynebacterium diphtheriae* was a *mitis* strain grown in the medium used for staphylococcus, with the further addition of pimelic acid and either β-alanine ($2 \times 10^{-5}M$) or pantothenate

* Leverhulme Research Fellow.

(8×10^{-9} *M* to 2×10^{-7} *M*) and with the omission of lactoflavin and ammonium salts. *Streptococcus haemolyticus* was grown in Medium III of McIlwain (1940*a*) containing mercaptoacetate.

All cultures contained the materials to be tested in 10 ml. of medium and were incubated at 37° in a 5 per cent. CO_2-air mixture, coli, typhoid, proteus and *C. diphtheriae* being in 50 ml. conical flasks, and staphylococcus and streptococcus in tubes.

Adequate control experiments with sulphite, aldehydes and aldehyde bisulphite compounds were carried out to ensure that the observed inhibitions were not due to products of decomposition of the amino-sulphonic acids (*cf.* also McIlwain, 1941).

2. INHIBITION BY α-AMINOSULPHONIC ACIDS.

The effects were investigated with amino-sulphonic acid concentrations of $1{\cdot}6 \times 10^{-5}$ *M* to 10^{-2} *M*. The following is a summary of the findings :

A. *Different organisms* are susceptible to different degrees (Table I), the inhibitions in general increasing with increasing nutritional requirements of the organism. Inhibition of streptococcus was similar to that of staphylococcus.

B. *Trained strains* of *Bact. typhosum* and of staphylococcus produced (Gladstone, 1937*a*, *b*) from the organisms used above and capable of dispensing with most added amino acids were not found to be inhibited. The difference is very marked in the case of staphylococcus, which is not known to differ from the normal strain except in its greater ability to synthesize amino acids : this suggests a correlation between aminosulphonic acid inhibition and amino acid, rather than general, metabolism.

C. *Removal of inhibitions by α-aminocarboxylic acids* :

1. *Proteus.*—The inhibitory effects with five different strains (Table II) showed no correlation in degree with general synthetic ability as represented by rate of growth. The addition of aminocarboxylic acids reduced the inhibitions, sometimes only by the aminocarboxylic acid of corresponding structure, and sometimes by others also.

The specificity of this reversal of inhibition was investigated with three organisms in the case of α-amino*iso*butane sulphonic acid. This compound was not found inhibitory when added to broth cultures of the organisms : constituents of the broth could reverse the inhibitions. β-Alanine, urea, aneurin, lactoflavin, adermin, uracil, pimelic acid, pantothenic acid ; lactate, succinate, α-ketoglutarate and glucose were without action on the inhibited growth. A group of α-aminocarboxylic acids, however, removed the inhibition entirely : aminocarboxylic acids become limiting factors in growth when aminosulphonic acids are added.

The effects of individual amino acids on inhibition of the laboratory strain of proteus by *a*-amino*iso*butanesulphonic acid are shown in Table III. Glycine and alanine partly remove the inhibition, but the most complete reversal is given by the corresponding amino acid, valine. It was found that a higher concentration of inhibitor required a higher concentration of amino acid for a similar reduction of its inhibition, though the effect of a given ratio of amino acid and aminosulphonic acid concentrations was not constant. The effects

TABLE I.—*Effect of α-Aminosulphonic Acids on Different Organisms.*

Organism.	α-Aminosulphonic acid added.	Molarity × 100.	Growth after (hours and days). 20–23.	27–30.	2.	3.	4.	5.
Bact. coli	0	..	++	+++	++++			
	Methane or *iso*pentane	1	?	++	++++			
	Ethane or *iso*butane	1	?	+	++++			
Proteus vulgaris (laboratory strain)	0	..	++	+++	++++			
	Methane	1	0	0	0	+++	++++	
	,,	0·2	0	+	+++	++++		
	Ethane and *iso*butane		See Table III.					
	*Iso*pentane	1	0	0	0	+++	++++	
	,,	0·2	0	0	?	++++		
	,,	0·04	0	?	++++			
	Phenylmethane	1	0	0	0	0	0	0
	,,	0·2	0	0	0	++	++++	
	,,	0·04	0	++	++++			
Bact. typhosum	0	..	++	++	+++	++++		
	Methane	1	0	0	0	0	0	
		0·2	0	?	?	+++	++++	
	Ethane	1	?	+	++	++++	++++	
Staphylococcus aureus	0	..	++	+++	++++			
	Methane or *iso*pentane	0·04	0	0	0	0	0	
	,, ,,	0·008	0	0	+	++++		
	,, ,,	0·0016	+	+++	++++			
	Ethane or *iso*butane	0·04	0	0	0	++++		
	,, ,,	0·008	0	0	++	++++		
	,, ,,	0·0016	+	++	++++			
	Phenylmethane	0·2	0	0	++	++++	++++	
	,,	0.04	0	0	+++	++++		
	,,	0·008	0	+	++++			

Note.—Plus signs are proportional to mass of growth, estimated visually.

TABLE II.—*Inhibition of Proteus Strains by α-Aminosulphonic Acids, and the Effects of α-Aminocarboxylic Acids.*

Strain.	α-Aminosulphonic acid added (0·002 M).	Aminocarboxylic acid added (0·01 M).	Growth after (days).				
			1.	2.	3.	4.	5.
N.C. 401 (*vulgaris*, N.Y.U.)	0, or ethane	0	0	+	++++		
	*iso*butane	0	0	0	0	0	0
	,,	Alanine	0	+	++++		
	,,	Valine	0	+	+++	++++	
N.C. 2100 (*vulgaris*, rat)	0	0	?	++	+++	++++	
	Ethane	0	0	0	+++	++++	
	,,	Alanine	?	++	++++		
	*Iso*butane	0	0	0	0	++++	
	,,	Glycine	0	?	+++	++++	
	,,	Valine	?	+++	++++		
N.C. 4199 (*vulgaris*, monkey)	0	0	?	+++	++++		
	Ethane	0	0	++	++++		
	,,	Alanine	?	+++	++++		
	*Iso*butane	0	0	0	0	0	0
	,,	Glycine or alanine	0	+++	++++		
	,,	Valine	?	+++	++++		
N.C. 3518 (*morgani*, Thjøtta)	0	0	+	+++	++++		
	Ethane	0	0	+++	++++		
	,,	Alanine	?	+++	++++		
	*Iso*butane	0	0	0	0	0	0
	,,	Glycine	0	0	+++	++++	
	,,	Valine	?	+++	++++		

Laboratory strain, see Table III.

TABLE III.—*Inhibition of Laboratory Strain of* Proteus vulgaris *by α-Aminosulphonic Acids, and the Effects of α-Aminocarboxylic Acids.*

α-Amino-sulphonic acid.	Molarity, × 100.	Aminocarboxylic acid.	Molarity, × 100.	Growth after (days). 1.	2.	3.	4.	7.
0	..	0	..	++	++++			
Methane	0·2	0	..	0	0	+++	+++	
,,	0·2	Glycine	0·1, 0·01	0	++	++++		
Ethane	1	0	..	0	0	+++	+++	
	0·2	0, or glycine, or valine	0·2 0·2	0	+++	+++	++++	
	0·04	0	..	?	++++			
,,	1, 0·2 or 0·04	Alanine	1	+	++++			
,,	1	,,	0·2	?	+++	++++		
,,	0·2, 0·04	,,	0·2	+	++++			
,,	1	,,	0·04	0	+++	+++	++++	
,,	0·2	,,	0·04	0	+++	++++		
,,	0·04	,,	0·04	?	++++			
*Iso*butane	1, or 0·2	0	..	0	0	0	0	0
	0·04	0, or leucine, or *nor*leucine	0·001 0·1, 0·01	0	0	+	++++	
	0·008	0	..	0	?	++++		
	0·0016	0	..	+	+++	++++		
	0·0032	0	..	++	++++			
,,	0·04	Glycine or alanine	0·1, 0·01	0	++	+++	++++	
,,	0·04	Leucine	0·1, 0·01	0	0	0	0	0
,,	1	Valine	1	0	0	++++		
,,	0·2 or 0·04	,,	1	?	++++			
,,	0·008 or less	,,	1	++	++++			
,,	1 or 0·2	,,	0·2	0	0	0	++	++++
,,	0·04	,,	0·2	0	++	+++	++++	
,,	0·008	,,	0·2	0	+++	++++		
,,	0·0016	,,	0·2	++	++++			
,,	1 or 0·2	,,	0·04	0	0	0	0	0
,,	0·04	,,	0·04	0	0	0	+++	++++
,,	0·008	,,	0·04	0	+	+++	++++	
,,	0·0016	,,	0·04	++	++++			

TABLE IV.—*Inhibition of Staphylococcus by α-Aminosulphonic Acids, and the Effects of Aminocarboxylic Acids.*

α-Aminosulphonic acid.	Molarity, × 100.	Aminocarboxylic acid.	Molarity, × 100.	Growth after (hours and days). 20–23.	27–30.	2.	3.
0	..	0	..	++	+++	++++	
Ethane	0·04	0	..	0	0	0	++++
,,	0·04	Glycine 1 or 0·2		++	+++	++++	
Butane	0·04	0, or alanine or β-alanine, 1; valine 0·2		0	0	0	++++
,,	0·04	Glycine 1; or 0·2		+	++	++++	
,,	0·04	Valine 1		+	++	++++	
Phenylmethane	0·2	0, or glycine, 0·2; β-alanine, 1 valine, 1; or phenylalanine, 0·2		0	0	++	++++
,,	0·2	Glycine, 1; or phenylalanine, 1		+	++	++++	

of amino acids on the inhibition caused by α-aminoethane sulphonic acid are similar. None of the organisms gave instance of specific reversal by particular amino acids.

2. *Staphylococcus* requires many amino acids for optimal growth (Gladstone, 1937*b*). Alanine is not one of the essential amino acids, and the effect of α-aminoethane sulphonic acid was therefore tested in the presence and absence of the amino acid. Addition of up to *M*/100 alanine did not affect the inhibition. Increase in concentration of other amino acids above the *M*/2000 of the medium did however reduce the inhibition ; this was notable with *M*/100 to *M*/500 glycine. α-Amino*iso*butane sulphonic acid could not be tested in the absence of valine, but by increasing the valine concentration to *M*/100, inhibition was considerably reduced. Glycine also reduced the inhibition ; β-alanine had no effect. *a*-Aminophenylmethane sulphonic acid was partly reversed by glycine and phenylalanine, which it may be said to resemble, but not by valine nor β-alanine. The effect of α-amino*iso*pentane sulphonic acid could not be tested in the absence of leucine, which is also essential, but increasing leucine concentration, and also that of valine, had no action ; a group of amino acids (alanine, *a*-aminobutyric acid, leucine, *iso*-leucine and *nor*leucine) did, however, reduce the inhibition.

It was sometimes found that particular concentrations of α-aminosulphonic acids accelerated growth of staphylococcus, especially if this was delayed through the concentration of a particular amino acid being suboptimal, though an aminosulphonic acid was never found to replace an essential amino acid altogether lacking. Such relationships have previously been noted with inhibitory compounds (McIlwain, 1940*b*).

3. CYSTEIC ACID, TAURINE AND TAURINE AMIDE.

These compounds were never found inhibitory. Cysteic acid was tested with coli, proteus and staphylococcus, and taurine and taurine amide with those organisms and also with a *mitis* strain of *Corynebacterium diphtheriae*, grown with limiting amounts of β-alanine or pantothenic acid. The compounds sometimes stimulated suboptimal growth.

4. DISCUSSION.

The hypothesis that aminosulphonic acids owe their inhibitory action to interference with aminocarboxylic acid metabolism has thus been justified by experiments with trained organisms and by reducing their action with aminocarboxylic acids. While α-aminocarboxylic acids as a class show considerable specificity in their reduction of the inhibitions, there is no specificity between the aminocarboxylic and aminosulphonic acids of corresponding structures. This is quite consistent with the general conception (Fildes, 1940) that such interference occurs at the surfaces of enzymes. Proteolytic enzymes, for example, are not specific to particular amino acids, and though quantitative differences exist in their rate of attack of peptides containing different amino acids, this is comparable to the different degrees of

inhibition and reversal recorded above. That the aminocarboxylic acid which best reduces the inhibition of a given aminosulphonic acid varies with different organisims is explained in terms of the different synthetic abilities and needs of the organisms, which produce their optimal quantities of amino acids to different degrees.

Instances of temporary inhibitions lasting for 2–4 days are frequent, and it was thought possible that the delayed growth might differ in its need for aminocarboxylic acids or in resistance to aminosulphonic acids. This was not found to be the case.

The lack of inhibitory power of other aminosulphonic derivatives investigated indicates the limitations of a working hypothesis based on structural analogy between metabolite and possible inhibitor. Production of a compound capable of interfering with a particular metabolite evidently demands consideration of properties of which structural formulae give only approximate indications.

My thanks are due to Dr. P. Fildes for suggestions in connection with this work, to Dr. G. P. Gladstone for cultures and details of the trained organisms, and to Mr. D. E. Hughes for valuable assistance.

SUMMARY.

α-Aminosulphonic acids delay or prevent growth, in chemically defined media, of a number of organisms, notably of proteus and staphylococcus. The inhibitions can be reduced or removed by the further addition of *a*-aminocarboxylic acids, which need not be those corresponding in structure to the inhibiting aminosulphonic acid; the reversing action is, however, specific in that among simple nutrients it is practically confined to α-aminocarboxylic acids. When staphylococcus is made independent of most added aminocarboxylic acids by training, α-aminosulphonic acids are no longer inhibitory. The results are considered to afford further instances of the blocking of enzyme reactions, essential to growth, by inhibitors which act on account of their structural similarity to normal substrates of the enzymes concerned. Compounds so related to essential metabolites are, however, not in all cases inhibitory, and can sometimes stimulate growth.

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A TRANSPLANTABLE MAMMARY FIBROADENOMA OF THE RAT SHOWING SARCOMATOUS CHANGES.

F. R. SELBIE.

From the Bland-Sutton Institute of Pathology, Middlesex Hospital, London, W. 1.

Received for publication May 29, 1941.

THE successful transplantation of a mammary fibroadenoma of the rat was first reported by Loeb and Fleisher in 1916, and since then a number of rat fibroadenomata have been propagated in series by several workers. This transplantable benign tumour is of great interest because, during propagation, it frequently shows great structural variations of which the most important is the appearance of new neoplastic properties in the connective tissue. In the present communication an account will be given of the experimental and histological findings obtained during the propagation of a rat fibroadenoma in which structural alterations were a notable feature.

MATERIAL AND METHODS.

The tumour strain on which this study has been made was kindly supplied by Dr. W. E. Gye, Director of the Imperial Cancer Research Fund. The tumour was then in its second passage from a spontaneous tumour found in the laboratory stock rats of that institute. Transplantation was performed by the subcutaneous inoculation of small pieces of tumour, about 5–10 mg. in weight, in both flanks of normal rats. The rats were usually two to four months old at the time of inoculation, and were bred from the strain in which the original spontaneous tumour was found. On a few occasions material for transplantation was obtained by biopsy so that the subsequent growth of the tumour could be observed, and in one rat (3A, No. 1) this method enabled two passages from each of the two tumours to be made at long intervals. After transplantation the rats were palpated at monthly intervals, and the tumours were charted in outline along with a measurement of the long axis of the tumour. In each passage the rats were numbered in the order that they showed palpable tumours.

EXPERIMENTAL RESULTS.

The tumour strain was transplanted into 8 rats in January, 1937, constituting passage 3A and the results of this passage and of the other 11 transplantations made in a total of 47 male and 74 female rats are shown in Fig. 1. Two fibroadenomata which appeared in Rat 3, Passage 3A and in Rat 11, Passage 5A, at 82 and 57 weeks after inoculation were probably spontaneous

tumours and are therefore omitted. It will be seen that the proportion of successful inoculations varies considerably from one passage to another. This variation in transplantability can be accounted for as due to variations in the tumour material and in the injected rats. The most important of these variations, the appearance of independent connective tissue tumours, is indicated in Fig. 1, and since this occurred in the tumour in Rat No. 2, Passage 4A, the tumours in Passages 5C, 6B and 6C are all purely connective tissue tumours and no longer represent the original tumour strain.

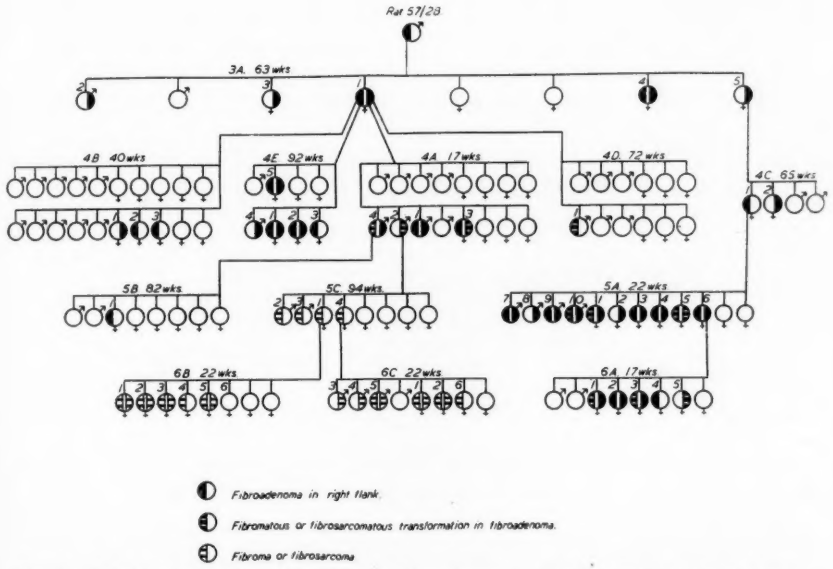


FIG. 1.—Results of transplantation of the rat fibroadenoma. Each transplantation is designated by a figure representing the generation or number of passages from the original spontaneous tumour and a letter signifying the series in that passage in order of time; *e.g.* 4C = 3rd series in 4th passage. The interval between transplantations is indicated by the number of weeks.

Transplantability.

Some of the factors influencing the success of transplantation of fibroadenomatous tumours are shown in Tables I and II, where the results of all passages except 5C, 6B and 6C are analysed. Table I shows that in general the proportion of successful inoculations decreases with the age of the parent tumour. This is in agreement with Heiman (1934), who found that young tumours gave the best takes. In Table II it is shown that the proportion of successes is much higher in female rats, 46 per cent., than in male rats, 28 per cent. This finding is characteristic of the rat fibroadenoma and indeed the tumours described by Robinson and Grauer (1932) and Oberling, Guérin and Guérin (1933) failed entirely to grow in male rats. Loeb and Fleisher (1916)

succeeded in transplanting their tumour in only one male rat. Heiman (1934), however, obtained 33 and 66 per cent. positive inoculations in male and female rats respectively, a similar result to that obtained in the present series. Heiman (1940*a*, 1940*b*) has also found that, in general, the percentage of takes is increased by injections of female sex hormone and decreased by injections of male sex hormones.

TABLE I.—*Effect of Age of Tumour Material on Success of Transplantation.*

Interval from previous passage (weeks).	Passages.	Number of rats inoculated.	Positive rats.	
			Number.	Per cent.
17–22	4A, 5A, 6A	36	19	53
40–65	3A, 4B, 4C	28	10	36
72–95	4D, 4E, 5B	28	7	25

TABLE II.—*Effect of Sex and Tumour Sensitivity of the Rats on Success of Transplantation.*

Passages 3A, 4A, 4B, 4C, 4D, 4E, 5A, 5B, 6A.

Sex.	Number of rats inoculated.	Number of rats positive in :			Total positive rats.	
		Right flank.	Left flank.	Both flanks.	Number.	Per cent.
Male	36	6	9	5	10	28
Female	56	20	20	14	26	46
Totals	92	26	29	19	..	..
Per cent.	..	28	32	21	..	..

The age of the parent tumour and the sex of the inoculated rat are, however, not the only factors influencing transplantation as can be seen from several exceptions in individual passages (Fig. 1). Another important factor can be deduced from the frequency with which successful inoculations coincide in both flanks of the rats (Table II). The proportion of takes to inoculations is 28 per cent. in the right flanks, and 32 per cent. in the left flanks, that is 30 per cent. in all. If the distribution of successful inoculations was controlled entirely by chance variations in the composition of the inoculated graft, the proportion of rats bearing two tumours would be about 9 per cent., but this proportion is actually 21 per cent., and is as high in the male as in the female rats. This high proportion of both male and female rats with tumours on both sides can only be due to the greater sensitivity of these rats to tumour transplantation. This varying sensitivity of rats to tumour growth has a great influence on the successful propagation of rat tumours, and it has been shown that susceptibility can be increased by selective breeding (Selbie, 1936*b*). This was shown to be a general susceptibility to rat tumours and not to a particular strain as has also been shown in mice, where bilateral inoculations of two different tumours tend to grow similarly according to the general susceptibility of the host (Selbie, 1936*a*).

When the tumour changed its character and became a purely connective tissue tumour in Passages 5C, 6B and 6C there were notable changes in its

transplantability (Fig. 1). The proportion of successful inoculations was much higher in rats of both sexes and was no lower in male than in female rats.

Growth Characters.

The rate of growth of the tumours, excluding the purely connective tissue tumours in passages 5C, 6B and 6C, was very slow and very variable. The average time taken for the tumours to become palpable was 18 weeks, with a range of 4 to 40 weeks in female rats, and 27 weeks, with a range of 15 to 40 weeks in male rats. After the tumours became palpable the average time taken to reach a long diameter of 2·5 cm. was 24 weeks, with a range of 9 to 39 weeks,

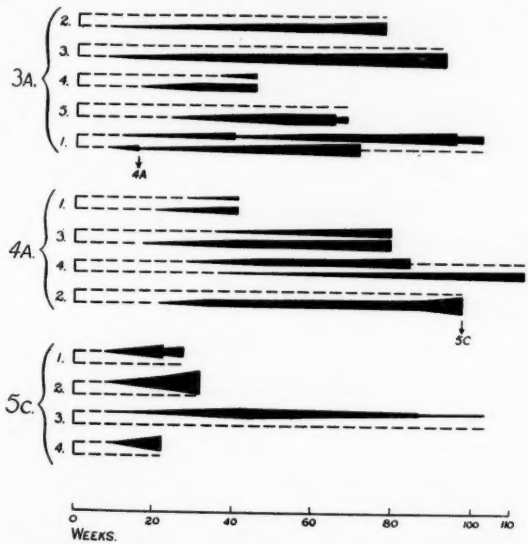


FIG. 2.—Growth of tumours in Passages 3A, 4A and 5C. Only tumour-bearing rats are shown. Each rat is numbered and the tumour grafts in the right and left flanks are represented by an upper and lower line respectively. So long as the graft shows no palpable growth it is represented by an interrupted line. The subsequent growth of tumours is represented by an expanding triangle, the width of which at any point is 1/25 of the long axis of the tumour at the number of weeks indicated. An interruption in a triangle indicates partial removal of the tumour by operation and cessation followed by an interrupted line signifies total removal without recurrence. The derivation of Passages 4A and 5C is indicated. Note the sudden expansion in the final stages of the growth of the tumour in Rat 2, Passage 4A and the continuance of this increased growth rate in 3 tumours in Passage 5C.

for 23 tumours in females, and 28 weeks, with a range of 8 to 69 weeks, for 9 tumours in males. The growth characters of the fibroadenomatous tumours in Passages 3A and 4A are shown graphically in Fig. 2. It will be seen that great differences in growth rate are also evident in pairs of tumours in one rat, as for example, in Rat 3, Passage 4A, where the tumour in the left flank became palpable at 22 weeks and attained a long diameter of 2·5 cm. in 35 weeks, whereas the right flank tumour did not reach these stages until 38

and 74 weeks after inoculation. Such differences in tumours in the same host were almost certainly due to variations in tumour structure, of which evidence will be given later.

The most important variation in the structure of these tumours, the appearance of fibrosarcomatous changes, was heralded in 3 tumours by a sudden increase in growth rate. As is shown in Fig. 2, this was observed in Rat 2, Passage 4A, at 82 weeks, after a period of 12 weeks suspended growth. At 82 weeks the tumour measured 4·0 by 3·5 cm. and by the end of the following 12 weeks, when the rat was killed, the tumour measured 7·0 by 5·5 cm. so that it had increased its volume 4 or 5 times. The two other tumours in which this occurred were in Rat 1, Passage 4D, and Rat 1, Passage 5A, and they showed approximately 3- and 4-fold increases on their previous growth rates after 62 weeks and 95 weeks respectively. Although the new growth rates were much less than that of established malignant transplantable rat tumours, they signified that a radical change in the structure of the tumours had taken place. At autopsy most of the tumour in Rat 2, Passage 4A, was seen to consist of fibromatous or fibrosarcomatous tissue, which was successfully transplanted in the right flanks of the rats in Passage 5C (Figs. 1 and 2). Other

EXPLANATION OF PLATES.

FIG. 3.—Tumour from the right flank of Rat 2, Passage 4E, at 67 weeks. The tumour is pericanalicular in structure and consists of acini and tubules around which the dense fibrous tissue is arranged in a concentric manner. × 30.

FIG. 4.—Tumour from the left flank of Rat 1, Passage 3A at 72 weeks. This part of the tumour is in active growth and is composed of mammary acini of normal appearance embedded in abundant well-differentiated fibrous tissue. Several mitotic figures can be seen in the epithelium and in the connective tissue. × 270.

FIG. 5.—Tumour from the right flank of Rat 1, Passage 3A at 95 weeks. Most of the acini are cystic and are lined by vacuolated epithelium. Many contain secretory products and desquamated epithelium. Some of the larger cysts are lined by flattened epithelium. × 60.

FIG. 6.—Tumour from the left flank of Rat 5, Passage 5A, at 111 weeks. There are cysts lined by epithelium several layers deep and there is desquamation of epithelial cells. Irregular acinar proliferation is also present, but there is no evidence of carcinomatous changes. Other parts of this tumour showed a great variety of epithelial alterations, including a large keratin cyst lined by squamous epithelium. × 150.

FIG. 7.—Tumour from the right flank of Rat 3, Passage 4A, at 77 weeks. The epithelium in this part of the tumour consists mostly of cysts lined by squamous epithelium, and filled with keratin. The figure also shows fibrous replacement of the epithelium (*cf.* Fig. 11). × 75.

FIG. 8.—Tumour from the left flank of Rat 1, Passage 4B, at 18 weeks. The epithelial tissue shows sebaceous metaplasia with the formation of cysts. × 100.

FIG. 9.—Tumour from the right flank of Rat 1, Passage 3A, at 40 weeks. A large duct is being distorted by nodular ingrowths of cellular connective tissue. × 45.

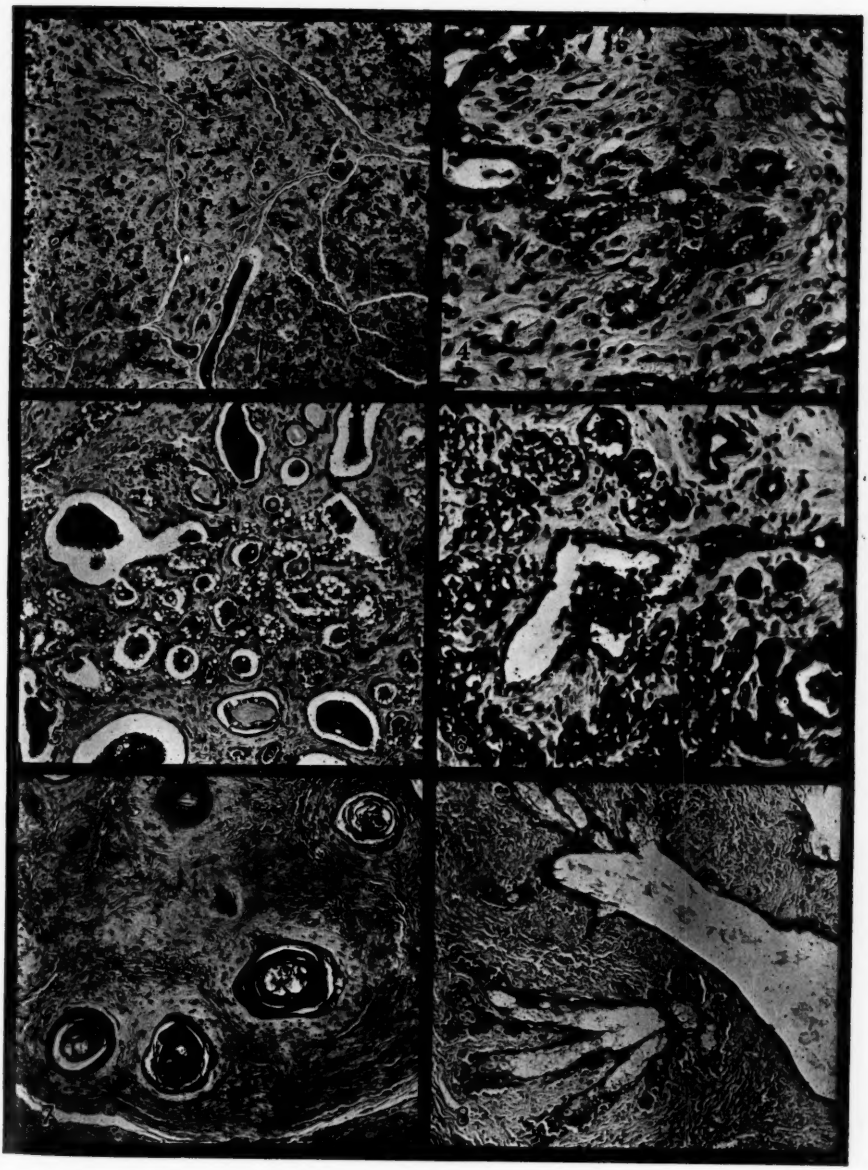
FIG. 10.—Another field of the section shown in Fig. 9. The nodular ingrowths of fibrous tissue have now produced the appearance of an intracanalicular fibroadenoma. × 30.

FIG. 11.—Tumour from the right flank of Rat 1, Passage 4D, at 80 weeks. The acinar tissue has atrophied and has been replaced by fibrous tissue, preserving the original arrangement of the tumour. In another part of this tumour there is complete loss of the original structure with the appearance of sarcomatous changes in the connective tissue. × 65.

FIG. 12.—Tumour from the left flank of Rat 2, Passage 4A, at 94 weeks. The epithelial tissue has disappeared and the original acellular fibrous tissue is being invaded by nodules of proliferating connective tissue cells. × 100.

FIG. 13.—Higher power of part of Fig. 12. The proliferating connective tissue cells are growing into the original fibrous tissue shown at the lower part of the figure. Two mitotic figures are shown. × 400.

FIG. 14.—Tumour from the right flank of Rat 2, Passage 6B, at 18 weeks. The tumour consists of fibroblastic cells with abundant collagen formation. Three mitotic figures are shown. × 400.



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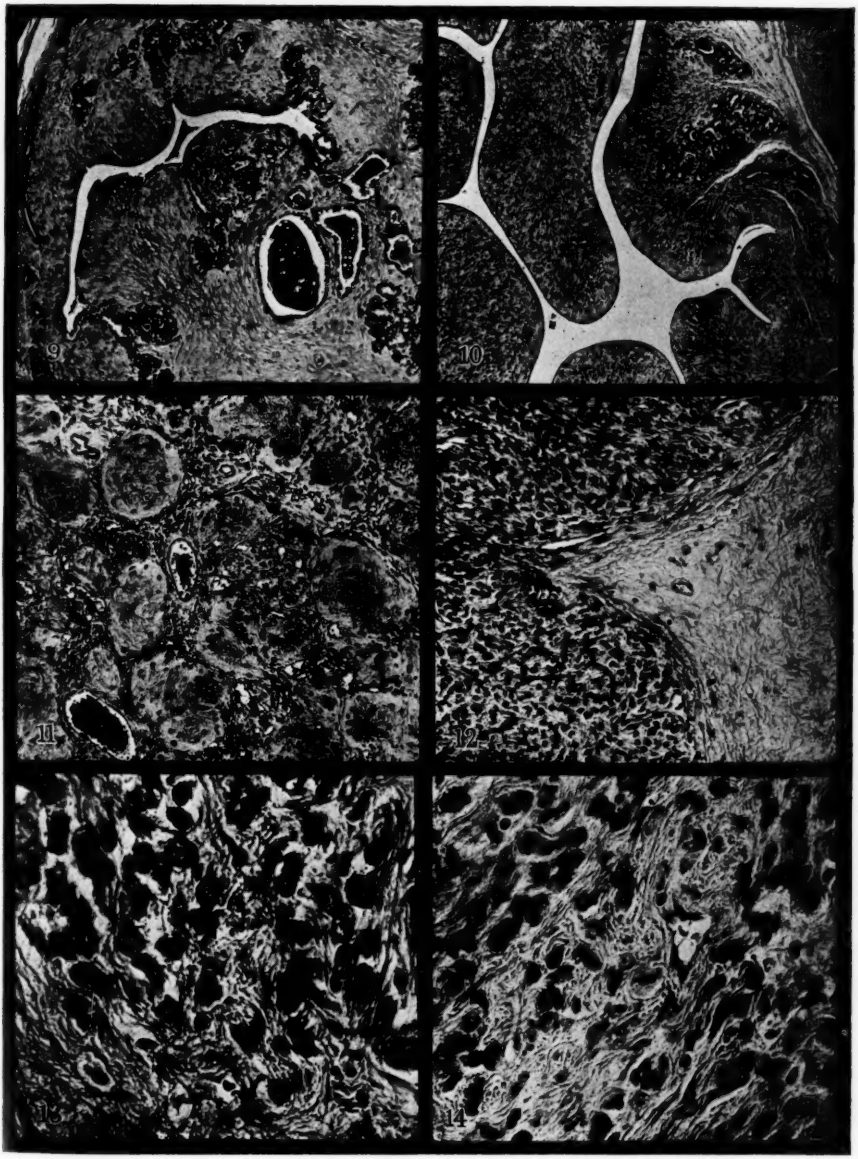

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parts of the tumour showed the structure of the original tumour, but grafts failed to grow when they were inoculated in the left flanks of the same rats. Transplantation of the connective tissue tumour yielded 3 rapidly growing tumours in Passage 5C (Fig. 2), 6 in Passage 6B and only 1 in Passage 6C. These 10 tumours became palpable at an average interval of 8 weeks and attained a long diameter of 2·5 cm. at an average of 5 weeks after becoming palpable. The remaining 12 tumours in these passages, and 4 tumours in a further passage from one of the tumours in Rat 2, Passage 6B, were hard fibromata of extremely slow growth. A passage was also made from the fibrosarcomatous tumour in Rat 1, Passage 5A, but yielded only one tumour which became palpable at 8 weeks and had completely regressed at 16 weeks.

From the above account of the transplantability and growth of this rat fibroadenoma it is evident that it shows characters of both normal and tumour tissue. The higher proportion of successful inoculations and more rapid growth in female rats indicate that the tumour is subject to hormonal influences and, in this respect, resembles normal mammary gland tissue. On the other hand, like other transplantable tumours its transplantability is controlled to some extent by the general susceptibility of both male and female rats. Furthermore, the susceptibility to hormonal influences apparently disappears when the tumour loses its epithelium and consists entirely of connective tissue.

HISTOLOGY.

Histological examinations have been made on 31 of the 55 tumours transplanted from fibroadenomatous material. Sections from each of the two tumours in Rat 1, Passage 3A, were obtained on two occasions so that, in all, 24 histological examinations have been made on tumours from female rats and 9 on tumours from male rats at periods ranging from 17 to 117 weeks after transplantation. Sections have also been made from 17 of the 22 connective tissue tumours in Passages 5C, 6B and 6C.

General Structure.

The original tumour strain presented the appearance of an intracanalicular fibroadenoma with numerous islets of glandular tissue deep in the abundant cellular connective tissue. The majority of the transplantation tumours, however, are of the more solid pericanalicular type and consist of acini or tubules embedded in wide bands of well differentiated fibrous tissue which is arranged round the epithelial tissue in a concentric manner (Figs. 3 and 4). The proportion of epithelial tissue to connective tissue varies in different tumours, and in some tumours where the acini or tubules are sparse the surrounding sheaths of dense fibrous tissue lie in an open fibrous tissue network.

Alterations in Epithelium.

Evidence of functional activity is frequently present in tumours from female rats. In part of the tumour removed from Rat 6, Passage 5A, during late pregnancy there is a great increase in the acini which are in a state of active secretion and the fibrous tissue is reduced to the proportion of a support-

ing stroma so that the tumour presents the appearance of a secreting adenoma. This effect of pregnancy on rat fibroadenomata has also been described by Oberling, Guérin and Guérin (1933) and Heiman and Krehbiel (1936), and Grauer and Robinson (1932) have found that lactation and involution in a rat fibroadenoma and in the normal mammary gland run an almost parallel course. Apart from the immediate effects of pregnancy, the results of continuous or repeated hormonal stimulation are apparent in about half the tumours from female rats at all stages of their growth. In these tumours a large proportion of the acini are lined by secreting epithelium and in 10 out of 20 of the tumours examined after 40 weeks growth in female rats there are numerous cystic acini lined by vacuolated epithelial cells (Fig. 5). In one tumour, examined 111 weeks after inoculation, there is a great variety in the lining epithelium of these cysts. Some are lined by secretory cells, some by flattened epithelium and others by epithelium several layers deep (Fig. 6). In association with the latter type of cystic acini there is an area of irregular acinar proliferation also shown in Fig. 6. These various glandular alterations, which would appear to be similar to those found in chronic mastitis in the human female breast, have also been noted in rat fibroadenomata by Oberling, Guérin and Guérin (1933, 1937), Heiman (1934), Heiman and Krehbiel (1936) and Emge (1938). None of these changes is present in tumours from male rats, where the glandular tissue is mainly tubular and shows no evidence of secretory activity.

Another type of alteration which has already been described in the epithelium of these rat fibroadenomata by Oberling, Guérin and Guérin (1938) is squamous or sebaceous metaplasia. In the present series squamous metaplasia is present in 2 tumours from female rats at 77 and 111 weeks and in 3 tumours from male rats at 41, 94 and 112 weeks. When fully differentiated the squamous acini take the form of cysts lined by keratinizing epithelium and sometimes filled with concentric layers of keratin (Fig. 7). In male rats the squamous metaplasia involves practically all the epithelium of the whole tumour, whereas in the female rats it is present only in small areas of tumours which are otherwise characterized by numerous secretory acini and cysts. Sebaceous metaplasia is seen in three tumours from female rats. In two examined at 65 and 92 weeks there are small numbers of acini showing sebaceous differentiation, and in the third, examined at 18 weeks, the epithelial tissue consists entirely of structures indistinguishable from sebaceous glands many of which can be seen to open into cysts lined by a thin layer of flattened epithelium (Fig. 8). This type of metaplasia is associated with numerous secreting acini and cysts in the first two tumours, and the third tumour was transplanted from a tumour showing similar secretory activity. It would therefore appear that a secretory stimulus favours the production of sebaceous metaplasia in these tumours.

Active growth of the epithelium can be seen in these tumours up to as long as 111 weeks after inoculation, but in many the epithelium becomes scanty and atrophic, and in 5 of the 20 tumours examined 70 to 117 weeks after the inoculation of fibroadenomatous grafts the epithelium has been entirely replaced or overgrown by fibrous tissue. Although these transplantation tumours show a very high degree of pleomorphism in the character and

arrangement of the epithelium, no changes suggestive of carcinoma have been found.

Alterations in Structure.

Formation of intracanalicular tumours.

The predominantly intracanalicular structure of the original tumour strain is reproduced in tumours from three male rats in Passage 4A at 41, 82 and 112 weeks and from one male rat in Passage 5A at 117 weeks, so that, including the original tumour, 5 out of the 10 tumours examined from male rats show this structure. In female rats, on the other hand, tumours showing an intracanalicular structure are seen only in Passage 3A, which was made from the original intracanalicular tumour; 3 of these, examined at 17, 44 and 44 weeks, are predominantly of this type, and 2, examined at 40, and 65 weeks, are pericanalicular tumours showing small intracanalicular nodules. Furthermore, later preparations at 72 and 92 weeks of the two tumours examined at 17 and 40 weeks from Rat 1 show the usual pericanalicular structure with no evidence of intracanalicular growth. It would therefore appear that in male rats this fibroadenoma tends to form an intracanalicular tumour which, on transplantation to female rats, reverts to the more solid pericanalicular form.

In one of the tumours from female rats the formation of intracanalicular nodules from the glandular tissue is well shown. This takes place by the differentiation of acini into ducts which become dilated and distorted by nodular ingrowths of the fibrous tissue (Figs. 9 and 10). Throughout the whole process the connective tissue is more cellular and more open in texture and is orientated perpendicularly to the epithelial surface of the ducts. Those structures appear to be formed by the preponderating activity of the connective tissue and their frequency in male rats would accord with the greater proliferative tendency of fibroblasts found by Heiman (1934) in fibroadenomata transplanted into young male rats. However, as the above observations in female rats show, this greater activity of the connective tissue can be carried over and can exist for some time in tumours transplanted to female rats.

The appearance of independent tumours in the connective tissue.

It has already been mentioned that there is a general tendency for the fibrous tissue to overgrow the epithelial elements in these fibroadenomata. This has taken place throughout the whole tumour in 5 and partially in 12 of the 20 tumours examined after 70 weeks' growth. In most cases the original structure of the tumour is lost, but in some tumours this overgrowth can be seen to take place by replacement of the epithelium. In pericanalicular tumours there is ingrowth of fibrous tissue from the periacinar bands to replace the acinar tissue (Fig. 11) and in intracanalicular nodules some of the inter-epithelial clefts become bridged over by fibrous tissue. In 12 of the 17 tumours showing fibrous overgrowth the connective tissue is wholly or partially hyaline and in some old tumours there are foci of calcification, but in none is there evidence of the formation of cartilage or bone. These regressive changes are

frequently seen in parts of tumours that are otherwise healthy in appearance and have been successfully transplanted.

The most striking change, however, in the connective tissue of tumours where the epithelium has wholly or partially disappeared is the occurrence of new tumours in 11 of the 19 fibroadenomatous tumours examined after 74 weeks (Fig. 1). The incidence of these changes is equal in male and female rats, and increases with the age of the fibroadenoma, being present in 6 of the 12 tumours examined between 74 and 100 weeks and in 5 of the 7 tumours examined between 100 and 117 weeks. In these fibroadenomata there are foci in which the connective tissue cells have become more numerous and have increased in size, giving the appearance of new growths with varying degrees of differentiation and apparent malignancy. Eight of the new tumours appear to be well differentiated cellular fibromata with little evidence of active growth. Histological characters suggestive of malignancy are seen in three tumours, from Rat 2, Passage 4A, Rat 1, Passage 4D, and Rat 1, Passage 5A, and these have already been described as showing a sudden increase in growth rate. These three tumours show similar histological features and Fig. 12 shows the periphery of the fibrosarcomatous part of the tumour in Rat 2, Passage 4A, where nodules of proliferating cells are invading the fibrous tissue of the original tumour. In Fig. 13 it can be seen that these cells are actively dividing fibroblasts with collagenous differentiation. The nature of this tumour is confirmed by the results of transplantation in Passages 5C, 6B and 6C, where all the tumours are of connective tissue and several of the more rapidly growing tumours have a similar fibrosarcomatous appearance (Fig. 14). Many of the passage tumours are acellular fibromata and in none of the apparently fibrosarcomatous tumours is there evidence of invasion of normal tissues or the formation of metastases. The malignancy of any of these tumours cannot therefore be established with certainty and their tendency to form acellular fibromata on transplantation indicates a variability not usually found in the more malignant fibrosarcomata.

DISCUSSION.

The appearance of sarcomatous changes seems to be an almost regular occurrence in rat fibroadenomata that have been sufficiently studied. It was at first attributed to the action of Sudan III by Umehara (1918) and was later described by Picco (1933) after injections of folliculin. It is now known from the work of Oberling, Guérin and Guérin (1933, 1935), Heiman (1934) and Emge (1938) that this transformation can take place spontaneously. Among the conditions favouring its appearance an important factor is time, as has been shown here and by Oberling, Guérin and Guérin (1935, 1937) and Emge (1938). Attempts to hasten the process by injections of Sudan III, tar (Heiman, 1934) and dibenzanthracene (Davis, Murphy and Emge, 1941) have all failed. Heiman (1940*a*, 1940*b*) was also unable to show any significant modifying effect after injections of oestrogenic, gonadotropic or somatotropic hormones, but with androgenic hormone he obtained a great increase in the incidence of fibromatous and sarcomatous alterations.

It has been shown in this communication that the appearance of new neoplastic properties in the connective tissue occurs only in tumours or parts of tumours where the epithelium has disappeared. This finding suggests the possibility that the fibrous tissue in these fibroadenomata has in some way been released from an influence exerted by the epithelium. Experimental evidence of this type of control in mixed tumours has been given by Russell (1910) in his account of the development of sarcoma in a mammary adenocarcinoma of the mouse. He found that the appearance of sarcoma in the stroma did not alter the carcinomatous rate of growth in sub-transplantations so long as carcinoma cells remained, but, when the sarcoma cells became quite freed from the carcinoma cells, their growth rate was very much increased. He concluded that the carcinomatous cells were capable of exerting a restraining influence upon the growth of the sarcoma cells, even although the latter might be more numerous and ultimately replace the carcinoma cells entirely. It would appear that there is a similar relationship between epithelium and fibrous tissue in the rat fibroadenomata. The structural modifications of the tumour in male rats also show the effect of a reduction in epithelial control, but to a smaller degree. Here the epithelium, inhibited by the unfavourable male environment, not only tends to form ducts, as has already been noted by Heiman (1940*b*), but also exerts less restraint on the connective tissue so that intracanalicular tumours are formed. The increased incidence of fibromata and sarcomata in rat fibroadenomata after injections of adrogenic hormone described by Heiman (1940*b*) can also be attributed to a release from some form of epithelial restraint for he also found that this hormone inhibits the glandular component.

It would therefore appear that sarcomatous changes in rat fibroadenomata can be attributed to the manifestation of the intrinsic malignant properties of the connective tissue component of the tumour, and that these properties are in some way restrained by the presence of the glandular tissue. The most likely explanation of the apparently controlling influence of the epithelium in fibroadenomatous tumours seems to be that the connective tissue is subservient to the needs of the epithelium in that it provides a supporting stroma. The fibrous tissue may therefore fail to display its full proliferative capacity in meeting this functional demand. On the other hand, the epithelium may exert an inhibiting action by an overwhelming demand for certain food or other essential factors or by a powerful organizing influence on the fibrous tumour tissue. In whatever way this may come about, it has been shown that by an alteration in environmental conditions the apparently benign fibromatous tissue of the fibroadenomata can assume the characters of malignant growth. Although none of these fibrosarcomata has yet been observed to form metastases, many have all the histological attributes of malignancy and some have shown infiltration of normal tissues (Oberling, Guérin and Guérin, 1935 ; Heiman, 1934).

Sarcomatous changes or increases in malignancy in connective tissue tumours have been frequently observed both in experimental work and in man. In the fowl sarcomata increases in malignancy are usually associated with, and may be attributed to, an increase in the activity of the virus agent (McIntosh and Selbie, 1939 ; Selbie and McIntosh, 1939). A similar associa-

tion in a mammalian tumour is suggested by the work of Parsons (1936, 1938) who found that the successful filtration of a chemically induced tumour of the mouse, Mal. sarcoma 1, was accompanied by an increase in transplantability and rate of growth. Whether the activity of a virus or other agent determines the malignancy of connective tissue tumours remains to be seen, but it does appear from the evidence presented here that an apparently benign fibromatous tumour may have inherent malignant properties and that its behaviour may be determined by extrinsic factors.

SUMMARY.

A description is given of the results of transplantation and the growth characters of a rat mammary fibroadenoma in which various alterations of the epithelial and connective tissue components were found.

The formation of cellular fibromata and sarcomata was observed in a number of fibroadenomata in which the glandular tissue had wholly or partially disappeared.

It is suggested that the glandular tissue of rat fibroadenomata has, in some way or another, a restraining influence on the intrinsic malignancy of the fibrous component.

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ACTIVE IMMUNIZATION AGAINST EPIDEMIC TYPHUS BY MEANS OF VACCINES PREPARED FROM ENDEMIC VIRUS.

M. RUIZ-CASTANEDA.

From the Typhus Laboratory of Mexico.

Received for publication May 24, 1941.

It was shown by Zinsser and Castaneda (1930, 1933) that active immunization of guinea-pigs with formalin-killed murine typhus rickettsiae conferred a higher degree of protection against the homologous virus than against the Breinl strain of European typhus virus. They reported that no more than 30 per cent. of vaccinated animals resisted infection by the latter strain. Verela, Parada and Ramos (1932) found that only 50 per cent. of similarly vaccinated animals withstood inoculation of Nicolle's Tunisian typhus virus. Mooser and Sparrow (1933) obtained no protection whatever against the heterologous strains. The antigens used by these various workers were prepared from the peritoneal washings of benzol treated typhus infected rats. Veintemillas (1939), using a richer antigen and multiple injections, protected all of his test animals against an epidemic strain of typhus virus isolated in Mexico.

Recently we have shown (Castaneda and Silva, 1939) that typhus as it occurs in Mexico City may be either of the endemic or epidemic type and that some of the strains of virus isolated have properties which would place them as intermediate between the murine and the classical European strains. These findings suggest possible modification of the murine type in the direction of the epidemic virus with continued passage in the human host. According to this hypothesis the epidemic strains would be regarded as variants of murine virus. We have discussed this question at some length (Castaneda, 1940) and expressed a belief that the experimental differences observed in guinea-pigs cannot be taken as evidence of fundamental antigenic differences in the rickettsiae of epidemic and endemic typhus. It would appear to us that the question is basically a quantitative one and that murine antigens of sufficient potency when displayed in adequate amounts confer protection against the heterologous strains.

Two new methods of preparing epidemic typhus vaccine in quantities requisite for large scale immunization have been described; that of Cox (1938) utilizes the yolk-sac of the developing chick, while that of Zinsser, Wei and Fitzpatrick (1937) employs a modified tissue culture technique. Since our report (Castaneda, 1939) on the infected rat lung as a source of large amounts of endemic rickettsial antigen methods have been devised for the mass production of typhus vaccine. The method is practical, rapid and inexpensive. In Mexico and elsewhere the use of the " lung vaccine " as an active immunizing agent has given encouraging results; these will be summarized together with the details of the preparation of the vaccine in the present paper.

Preparation of the "Lung Vaccine."

The virus used in the preparation of the vaccines described in this report is the "L" strain obtained from a typhus patient in Mexico City in 1936. It has the properties of murine virus and therefore grows readily in the guinea-pig tunica vaginalis and in the peritoneum of X-rayed rats. Tunica washings taken from guinea-pigs killed on the 6th day after intraperitoneal inoculation are injected into the abdominal cavity of X-rayed rats.* Animals may become considerably dehydrated during the last day or two of infection; this may be offset somewhat by the use of large animals and subcutaneous saline injections. Usually rats dying on the 5th day are optimum, but those killed on the 4th and 6th days may be satisfactory for passage virus. This can be determined by the numbers of rickettsiae revealed in stained smears of the peritoneal exudate. Five to 10 c.c. of 0·5 per cent. sodium citrate, according to the abundance of rickettsiae, are used to wash the peritoneum. The washings are then collected and centrifuged at low speed to remove gross particles. 0·2 c.c. of the supernatant is introduced intranasally in mice anaesthetized with a 1 : 3 chloroform-ether mixture. Mice inoculated in this manner generally die or are moribund in 72 hours. The lungs are removed aseptically and impression smears prepared from cut surfaces. Smears are stained by Giemsa's or Macchiavello's stain, or by our own methylene-blue safranin method which permits ready differentiation of rickettsiae and bacteria. For routine staining in vaccine work we have employed simple staining with a mixture of 5 c.c. of a 1 per cent. alcoholic solution of methyl-violet diluted in pH 7·5 phosphate buffer solution containing 5 per cent. formalin. Lungs shown to be rich in rickettsiae and free of bacteria by staining are pooled and emulsified by grinding with powdered pyrex glass and citrate solution, 5 to 7 c.c. being used for each pair of lungs. The tissue emulsion is lightly centrifuged at low speed to remove gross particles and the supernatant checked microscopically for the richness and purity of the rickettsial suspension.

Lung emulsions prepared in the above manner usually contain an abundance of rickettsiae and are suitable for passage by intranasal instillation in rats. 0·5 to 1 c.c. doses are used, depending upon the size of the animal. Deaths occurring after 48 and some after 72 hours are commonly due to bacterial pneumonia. The lungs of the remaining rats that are acutely ill or moribund after 72 hours are suitable for production of vaccine, as may be the lungs of animals found dead at this time. The lungs are removed aseptically, checked by microscopic examination and if found free of bacteria are emulsified in an electric homogenizer, such as the Waring Blender. 250 c.c. of 1 per cent. phenol in saline solution are added to the lungs of 5 animals. The emulsion is allowed to stand at room temperature over night, during which time the phenol precipitates the bulk of the tissue proteins, but leaves most of the rickettsiae in suspension. The following day the emulsion is centrifuged at 1500 r.p.m. for 20 minutes in 250 c.c. glass bottles. The supernatant is decanted and saved. The sediment is taken up in a small volume of diluent,

* The irradiation must be given on the same day as the inoculation. We have found 800 international r-units at 150 KV and 15 MA with the tube 40 cm. from the animals satisfactory. Knowing the time required to give this amount of irradiation the dosage can be varied so as to give the best results; this must be determined in each individual laboratory.

rehomogenized and then centrifuged as before. This process may be repeated two to three times, the supernatant being saved each time and pooled with the decanted rickettsial suspension of the original lung emulsion. The pooled supernatants are then spun in a high-speed angle centrifuge until microscopic examination of the supernatant fluid shows it to be free of rickettsiae. The optimum time for thorough sedimentation of the organisms must be determined by trial runs, since it will be governed by the available speed and tube-angle of the centrifuge and local conditions, such as altitude. The supernatant fluid is now discarded and the walls of the tubes or bottles carefully scraped with a rubber-tipped glass rod and washed down with a small amount of diluent to remove adherent antigen. The rickettsiae thus obtained can now be resuspended by grinding in a mortar with glass powder. Sufficient diluent is added, so that the crude vaccine represents the rickettsiae recovered from one pair of lungs suspended in 5 c.c. This product which has a slight brownish colour is held in the refrigerator for one week before making sterility tests.* Sterile batches are then centrifuged at low speed to remove any accumulated precipitates and the sediments are washed and centrifuged as many times as necessary. Preliminary standardization is made nephelometrically, however, depending upon the potency tests. The vaccine may be diluted to the required standard to be followed by potency tests which are carried out according to recent recommendations of the U.S. Public Health Service.† The required antigenic content of the vaccine can be readily adjusted. For large scale field immunization we have found it convenient to preserve the vaccine with formol, for which the phenolized rickettsiae suspensions are centrifuged at high speed in an angle centrifuge and the sediment resuspended in 0·2 per cent. formalin to a concentration of antigen which can be diluted 1 : 10 immediately before use. However, concentrations as high as 100 billion rickettsiae per c.c. can be obtained. For human immunization 0·5 to 1·0 c.c. doses, having a concentration of 3 billion per c.c. are well tolerated, but for practical purposes vaccines containing 1 billion per c.c. have been found to be satisfactory. As a general rule three doses are given : 0·5, 1·0 and 1·0 c.c., with a 7-day interval between injections.

Because of the frequency of bacterial contaminants and pneumonia it is not practical to attempt lung passage in rats, hence we have found it necessary to build up the potency of the inoculum for the rat lung by the guinea-pig-rat-mouse passage sequence described.

Results of Vaccination with "Lung Vaccine."

In a previous report (Castaneda, 1939) we indicated that small amounts of lung-vaccine prepared in the above-described manner protected guinea-pigs against infection by the "L" strain of murine typhus virus. This has been confirmed by Hudson (1940*b*), who found that as little as 0·2 c.c. given subcutaneously conferred a substantial degree of immunity. In titrating various

* The vaccine at this stage has been found by Hudson (1940*a*) to be a satisfactory antigen for diagnostic agglutination tests. After the addition of 0·2 per cent. formalin the rickettsiae become inagglutinable unless heated to 70° C. for 30 minutes.

† The method of standardization not yet published requires a potency test showing 75 per cent. protection against a dose of epidemic typhus virus capable to produce infection in 75 per cent. of normal guinea-pigs.

lots of lung-vaccine and using the homologous " L " strain as test virus we have determined that the vaccine prepared from the tissues of one rat may contain from 4000 to 10,000 guinea-pig-immunizing doses. Using a vaccine having a turbidity of 2 of the McFarland scale and prepared in our laboratories, Finlayson and Grobler (1940) reported successful protection of guinea-pigs against infection by both endemic and epidemic virus from South Africa. They observed that a single 1 c.c. dose was sufficient to protect against the endemic strains, whereas 2 such doses were required when epidemic strains were used as test virus. In a later report Finlayson (1940) found that when

TABLE I.—*Active Immunization Against Epidemic Typhus with Lung-vaccine.**

Guinea-pig No.	Concentration of the vaccine.	Number of doses.	Total amount given. c.c.	Days of fever (40° C. and over).	Results.
1	No. 2 McFarland (formalinized)	3	3	0	Protected.
2	Ditto	3	3	1	,,
3	,,	2	2	0	,,
4	,,	2	2	1	,,
5	,,	2	2	0	,,
6	,,	2	2	2	Partial protection.
7	,,	1	1	5	Not protected.
8	,,	1	1	9	,, ,,
9	Control	..	..	8	Epidemic typhus.
10	,,	..	..	7	,, ,,
A	No. 3 McFarland (formalinized)	3	3	1	Protected.
B	Ditto	3	3	0	,,
C	,,	3	3	0	,,
D	,,	2	2	1	,,
E	,,	2	2	0	,,
F	,,	2	2	0	,,
G	,,	1	1	3	Partial protection.
H	,,	1	1	3	,, ,,
I	,,	1	1	7	Not protected.
J	Control	..	..	10	Epidemic typhus.
K	,,	..	..	11	,, ,,
1A	No. 2 McFarland (phenolized)	2	1	3	Partial protection.
2A	Ditto	2	½	3	,, ,,
3A	(formalinized)	2	1	3	,, ,,
4A	Ditto	2	½	2	,, ,,
5A	Control	..	..	6	Moderate typhus.
6A	,,	..	..	11	Epidemic typhus.
1B	No. 1 McFarland (phenolized)	3	3	3	Partial protection.
2B	Ditto	3	3	0	Protected
3B	,,	3	3	0	,,
4B	(formalinized)	3	3	1	,,
5B	Ditto	3	3	3	Partial protection.
6B	,,	3	3	4	,, ,,
7B	Control	..	..	7	Epidemic typhus.
8B	,,	..	..	7	,, ,,

* The dose of virus used in these experiments was 0·2 g. of brain of an infected guinea-pig killed on the fifth day of fever and it has been found sufficient to produce 100 per cent. infection in normal guinea-pigs.

the vaccine was concentrated to one half its original volume by precipitation with alum animals were protected against the epidemic strain by a single 1 c.c. dose.

Because of the practical importance of the problem we have attempted to determine the minimum quantity of murine lung-vaccine necessary to protect against classical European typhus. The Breinl strain was used at test virus. The dose required to produce certain infection in all unvaccinated controls represented 0·2 gm. of infected guinea-pig brain prepared as a lightly centrifuges emulsion. The vaccine was standardized by the nephelometric method and was administered in 1 c.c. doses at weekly intervals. Representative results of our tests are set forth in Table I.

Certain quantitative relationships become apparent from a study of the above results. It will be seen that the 14 animals considered protected received an average dose of 2·78 c.c. of lung-vaccine equivalent to a turbidity No. 2 of the McFarland scale and had an average of 0·35 days of fever. On the other hand, the 10 animals regarded as partially protected received an average dose of 1·25 c.c. and had an average of 2·9 days of fever, while the 3 unprotected guinea-pigs had an average dose of 1·15 c.c. and 7 days of fever.

The treatment of guinea-pigs with a vaccine of high antigenic content (series A to K), shows that even in a single 1 c.c. dose the animals acquire a considerable degree of protection. However, it seems necessary to multiply the doses with vaccines of lower rickettsiae content.

DISCUSSION.

The striking difference in the immunizing capacity of our murine lung-vaccine as indicated by the response of vaccinated animals when tested with endemic and epidemic strains of typhus virus can be explained to a certain extent by a consideration of the nature of the infections caused in guinea-pigs by these two types of virus. The endemic strains tend to grow rapidly in the tunica vaginalis and to produce local reactions of considerable intensity. The appearance of antibodies such as opsonins as early as 96 hours after inoculation suggest that the development of localized lesion may accelerate the process of immunization ; thus small doses of vaccine seem to be sufficient to start a process of immunity reinforced upon subsequent exposure to homologous virus. Epidemic strains rarely provoke local reactions and usually result in rapidly generalized infections of longer duration and greater severity with critical localization in the brain. Under such circumstances small doses of vaccine are less likely to be effective in preventing infection under test conditions.

Whether or not there are essential antigenic differences between endemic and epidemic typhus viruses it is impossible to say from the information at hand. It is clear, however, from the present evidence that such differences are not great enough to justify disregard of vaccines prepared from endemic strains as practical immunizing agents against infection by the classic strains of typhus virus. The murine lung-vaccine when used in adequate amounts gives rise to a definite degree of immunity. In this connection it is of interest to refer to the results of Durand and Giraud (1940), who prepared an epidemic

type of mouse lung-vaccine. They found that single doses of their vaccine were insufficient to protect against infection by the homologous strain of virus ; two or three doses were required to give satisfactory immunity in test animals.

SUMMARY.

A practical method for the preparation of large quantities of typhus vaccine from the lungs of infected rats is described.

Evidence is presented to show that lung-vaccine prepared from an endemic strain of typhus virus will protect against infection by the classic strain of European typhus.

Certain relationships between the endemic and epidemic strains of typhus virus are briefly discussed.

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THE OXYGEN-LABILE HAEMOLYSIN OR θ TOXIN OF *CLOSTRIDIUM WELCHII*.

E. W. TODD.

From the Belmont Laboratories (London County Council), Sutton, Surrey.

Received for publication May 30, 1941.

NEILL (1926) showed that filtrates from cultures of *Cl. welchii* contain an oxygen-labile haemolysin which is inactivated by oxidation and reactivated by reduction.

Macfarlane, Oakley and Anderson (1941) introduced the lecitho-vitellin reaction for titrating the *Cl. welchii* α toxin of Glenny *et al.* (1933). This reaction, which gives parallel results with the lethal and haemolytic tests, depends on the appearance of opalescence in a solution of egg-yolk on incubation

with α toxin. It is due to the liberation of free fat and it occurs only in the presence of ionized calcium.

Todd (1934) showed that the oxygen-labile haemolysins of streptococci, pneumococci and tetanus bacilli are serologically related to each other. Thus high titred antistreptolysin O sera (Todd, 1938) neutralize in high dilution each of the three oxygen labile haemolysins. There is not, however, a constant ratio between the neutralizing doses of sera for the three haemolysins when a number of different sera are tested.

The haemolysins of *Cl. welchii* have now been examined with special reference to (1) oxygen lability, (2) relation to α toxin, (3) serological relation to streptolysin O, (4) relation to θ toxin.

Oxygen labile haemolysin of Cl. welchii *and its relation to α toxin.*

A number of different toxins of *Cl. welchii*, prepared from the Type A strain S 107, were titrated before and after reduction with sodium hydrosulphite for haemolysis of rabbits' blood cells. The α toxin was also titrated at the same time by the lecitho-vitellin reaction. In the majority of instances reduction caused no alteration of haemolytic titre ; in a few cases, however, a considerably increased haemolytic activity was observed without any corresponding increase in the titre of the egg-yolk reaction. An example of this effect of oxidation and reduction is given in Table I.

Although the egg reaction, which indicates the titre of α toxin gave constant readings with 0·01 c.c. of filtrate yet the haemolytic titre varied according to the state of oxidation or reduction. The fresh filtrate, reduced by bacterial metabolism, had a higher titre for red cells than for lecithin. After oxidation by storage in the cold the haemolytic and egg reactions gave almost identical readings. Reduction with sodium hydrosulphite restored the haemolytic activity almost to its original level. This shows that the filtrate contained an oxygen labile haemolysin in addition to the more stable haemolytic α toxin.

Serological relationship of the oxygen labile haemolysin of Cl. welchii *and streptolysin O.*

Since antistreptolysin O is known to neutralize the oxygen labile haemolysins of streptococci, pneumococci and tetanus bacilli it was interesting to see whether it would also neutralize the oxygen labile haemolysin of *Cl. welchii*. Neutralization experiments were therefore carried out with high titred antistreptolysin O serum against oxygen-labile *Cl. welchii* haemolysin. Haemolysis by α toxin, which is not neutralized by antistreptolysin O, was excluded by using a filtrate in which the oxygen labile haemolysin had a higher titre than the α toxin. Thus in Table I, one m.h.d. was contained in 0·0005 c.c. of the fresh untreated toxin, but at least ten times more toxin was required to produce haemolysis attributable to α toxin. Antistreptolysin O serum completely neutralized one m.h.d. of the fresh untreated toxin and it also continued to cause complete neutralization with increasing doses of toxin until the point was reached where haemolysis by α toxin occurred in spite of the presence of antistreptolysin O.

All the haemolysins which are known to be subject to reversible oxidation

TABLE I.—*Effect of Oxidation and Reduction on the Oxygen Labile Haemolysin of* Cl. welchii.

Condition of toxin and dates of tests.		Volume of toxin c.c.											
		0·5.	0·2.	0·1.	0·05.	0·02.	0·01.	0·005.	0·002.	0·001.	0·0005.	0·0002.	0·0001
1. Fresh toxin untreated 31.x.40.	Haemolysis	C	C	C	C	C	C	C	C	+++	++	tr	O
2. ,, ,, ,, ,,	Egg reaction	F	F	F	F	F	op	O	O	O	O	O	O
3. Stored ,, ,, 5.xi.40	Haemolysis	C	C	C	C	+++	++	+	O	O	–	–	–
4. ,, ,, ,, ,,	Egg reaction	F	F	F	F	F	op	O	O	O	–	–	–
5. Stored toxin untreated 27.xi.40.	Haemolysis	C	C	C	++++	+++	++	+	O	O	–	–	–
6. ,, ,, ,, ,,	Egg reaction	F	F	F	F	F	op	O	O	O	–	–	–
7. Stored toxin reduced 27.xi.40.	Haemolysis	C	C	C	C	C	C	C	+++	++	+	O	O
8. ,, ,, ,, ,,	Egg reaction	F	F	F	F	F	op	O	O	O	O	O	O

C = complete haemolysis; ++ = half haemolysis; F = flocculation; op = opalescence; – = not done;
O = no haemolysis or opalescence.

and reduction, namely those of streptococci, pneumococci, *Cl. tetani* and *Cl. welchii* are, therefore neutralized by high titred antistreptolysin O serum in high dilution.

In order to investigate the converse process of neutralization of streptolysin O by *Cl. welchii* antitoxin the sera of three horses which were being immunized with *Cl. welchii* toxin from strain S 107 were tested at weekly intervals for the homologous α antitoxin and for the heterologous antistreptolysin O. This was controlled by similar neutralization tests on bleedings from horses immunized with haemolytic toxins of *Cl. septique* and *Cl. oedematiens* which were not subject to reversible oxidation and reduction and which were not neutralized by antistreptolysin O. All the horses immunized with *Cl. welchii* toxin and none of the control horses showed a marked rise in antistreptolysin O titre during the early stages of immunization. These results are summarized in Table II.

TABLE II.—*Antistreptolysisn O Titres of Sera from Horses Undergoing Immunization with Various Haemolytic Toxins.*

Toxin used as antigen.	Antistreptolysin O units per c.c. of serum.		
	Before immunization.	During early immunization with small doses of toxin.	During later immunization with larger doses of toxin.
Welchii	125	10,000	5,000
,,	50	2,500	2,000
,,	100	20,000	10,000
Septique	40	20	20
,,	20	60	60
Oedematiens	40	100	100
,,	60	60	60

The remarkably rapid increase in the titre of antistreptolysin O which may occur during immunization with *Cl. welchii* toxin is shown in Table III. In this case a total dosage of 30 c.c. of toxin caused a rise of antistreptolysin O titre from 125 to 10,000 units per c.c. of serum in eleven days. In each of the three horses there was an early steep rise in antistreptolysin O which then gradually decreased as the α antitoxin increased, so that curves of the two antitoxins always crossed as in Table III.

It has thus been demonstrated that sera from horses immunized with *Cl. welchii* toxin neutralize streptolysin O. Conversely the sera of horses immunized with streptolysin O neutralize the oxygen-labile haemolysin of *Cl. welchii* as well as the other oxygen-labile haemolysins. There are, however, indications that the oxygen-labile haemolysins though closely related are not identical.

The ratios of doses of sera required to neutralize two oxygen-labile haemolysins.

Sera from horses undergoing immunization with streptococcus toxin and with *Cl. welchii* toxin were titrated against the homologous and the heterologous oxygen-labile haemolysins. The neutralizing action of concentrated

TABLE III.—*Details of Immunization of Horse with* Cl. welchii *Toxin.*

Date.	Dose of toxin c.c.	*Cl. welchii* α antitoxin units per c.c. of serum.	Antistreptolysin O units per c.c. of serum.
29.v.40	..	>1/5 <1/2	125
28.vi.40	5	..	..
1.vii.40	5	..	..
4.vii.40	10	..	..
6.vii.40	10	..	..
9.vii.40	15	80	10,000
11.vii.40	20	..	..
13.vii.40	20	..	..
15.vii.40	30	170	8,000
18.vii.40	30	..	..
20.vii.40	40	..	..
22.vii.40	40	400	8,000
24.vii.40	50	..	..
26.vii.40	50	..	..
29.vii.40	75	500	6,000
31.vii.40	75	..	..
2.viii.40	100	..	..
5.viii.40	150	600	5,000
7.viii.40	150	..	..
9.viii.40	150	..	..
12.viii.40	..	800	4,000

sera, of cholesterol (1939) and of novarsenobillon on the two lysins was also investigated. Two toxins were used throughout the experiment; and their reduction was maintained by storage in the cold under vaseline after adding 0·1 per cent. sodium hydrosulphite. The doses of the lysins which caused 80 per cent. haemolysis of a constant dose of washed rabbit blood cells were first determined. These doses were then used in all the neutralization experiments against varying quantities of serum on a 20 per cent. range.

Table IV shows that the two lysins were indistinguishable by neutralization tests with high titred sera. In every case the streptolysin O was neutralized by less serum than the *Cl. welchii* haemolysin; this difference was, on the average, three times greater with streptococcus serum than with *Cl. welchii* serum. Cholesterol had an effect on the two lysins which was quantitatively similar to that of the sera being more effective against streptolysin O than against *Cl. welchii* haemolysin. On the other hand, neo-salvarsan was definitely more effective against streptolysin O. The considerable variation in the ratios of the neutralizing doses of sera for the two lysins and the selective neutralizing action of neo-salvarsan seem to indicate that the two lysins are not identical substances although closely related.

The relation of Cl. welchii *oxygen-labile haemolysin to θ toxin.*

By exchanging sera with Dr. A. T. Glenny it has now been established that the oxygen-labile haemolysin of *Cl. welchii* is the *θ* toxin mentioned by Macfarlane, Oakley and Anderson (1941).

TABLE IV.—*The Neutralizing Doses of Streptococcus and* Cl. welchii *Antitoxins for the Homologous and Heterologous Oxygen-labile Haemolysins.*

Description of serum and dates of bleedings.	Dose of serum which neutralized the oxygen-labile haemolysin of: Streptococcus.	*Cl. welchii*.	Ratio of doses.
Streptococcus serum Horse 64 bled 21.xi.40	0·00005	0·00016	3·2
,, ,, ,, 64 ,, 19.xii.40	0·00012	0·00032	2·7
,, ,, ,, 64 ,, 10.iv.41	0·00008	0·00025	3·1
,, ,, ,, 65 ,, 21.xi.40	0·00008	0·0008	10·0
,, ,, ,, 65 ,, 19.xii.40	0·00012	0·0008	6·7
,, ,, ,, 65 ,, 10.iv.41	0·0001	0·001	10·0
,, ,, concentrated pseudoglobulin	0·0000025	0·000063	25·2
,, ,, ,, "digest" process	0·00002	0·0002	10·0
,, ,, ,, ,, ,,	0·00008	0·0008	10·0
Average for streptococcus antitoxins	..	..	9·0
Cl. welchii serum, Horse 50 bled 15.vii.40	0·000016	0·00012	7·5
,, ,, ,, ,, 50 ,, 22.vii.40	0·000016	0·00012	7·5
,, ,, ,, ,, 50 ,, 10.iii.41	0·00005	0·000063	1·3
,, ,, ,, ,, 56 ,, 22.vii.40	0·00005	0·0002	4·0
,, ,, ,, ,, 56 ,, 10.iii.41	0·000032	0·00005	1·6
,, ,, ,, ,, 70 ,, 26.viii.40	0·00016	0·00025	1·6
,, ,, ,, ,, 70 ,, 9.ix.40	0·0000063	0·000008	1·3
,, ,, ,, ,, 70 ,, 28.x.40	0·00001	0·000012	1·2
,, ,, ,, concentrated pseudoglobulin	0·000012	0·000016	1·3
Average for *welchii* antitoxins	..	..	3·0
Cholesterol	1 in 1,600,000	1 in 800,000	2·0
Neo-salvarsan	1 in 4,000	1 in 16,000	0·3

DISCUSSION.

Four bacterial haemolysins, namely those of streptococci, pneumococci, *Cl. welchii* and *Cl. tetani*, which were originally shown by Neill (1926) and by Neill and Mallory (1926) to be subject to reversible oxidation and reduction, are all serologically related to each other. They are all neutralized by high titred antistreptolysin O sera; conversely *Cl. welchii* θ antitoxin neutralizes streptolysin O. The action of the antibodies of tetanolysin and of pneumococcus haemolysin on the other oxygen-labile haemolysins has not yet been investigated. The haemolysins of staphylococci, *Cl. septique* and *Cl. oedematiens* are not reactivated by reduction and they are not neutralized by the antibodies of the oxygen-labile haemolysins.

All Griffith's types of haemolytic streptococci which belong to Lancefield's group A produce, in addition to streptolysin O, a second haemolysin, streptolysin S (Todd, 1939). It is serologically distinct from streptolysin O, it is not reactivated by reduction and, like streptolysin O it does not give a positive lecitho-vitellin reaction. Group C haemolytic streptococci of human origin and Group G strains both form oxygen-labile haemolysin which is indistinguishable from that of group A strains. In addition they also form haemolysins which are not oxygen-labile and are not neutralized by antistreptolysin O. Haemolytic streptococci of groups B, D, E, F, H, and K form haemolysins which are not reactivated by reduction and are not neutralized by antistreptolysin O. The two haemolysins formed by *Cl. welchii* type A have the

following characteristics. The α toxin is not oxygen-labile, it is not neutralized by antistreptolysin O and it gives a positive lecitho-vitellin reaction. The θ toxin is oxygen labile, it is neutralized by antistreptolysin O and it does not give a positive egg reaction. Pneumococci and *Cl. tetani*, so far as is known, produce oxygen-labile haemolysins only, there being no evidence at present of a second haemolysin as in the case of streptococci and *Cl. welchii*. A group of bacterial haemolysins is therefore distinguishable by reversible oxygen sensitivity and serological affinity.

Cross neutralization of the oxygen-labile haemolysins by heterologous sera seems to be dependent on the use of high titred sera. If low titred sera, such as those derived from natural infections, are used cross neutralization is much less evident. This is probably another expression of the phenomenon of irregular ratios between the neutralizing doses of high titred sera for different lysins.

The part played by the oxygen-labile haemolysins in pathology is uncertain. Recent experiments which are not yet complete seem to show that former failures to demonstrate any effect of streptolysin O on leucocytes were due to the presence of red cells mixed with the leucocytes which were being tested. Under these conditions the red cells are haemolysed and inactivate the haemolysin before it has time to act on the leucocytes. Streptolysin O and *Cl. welchii* θ toxin appear to be definite leucocidins when tested on leucocytes free from red cells. It is possible that the other oxygen-labile haemolysins have a similar leucocidin action.

SUMMARY.

1. The oxygen-labile haemolysin or θ toxin of *Cl. welchii* has the following characteristics. It is inactivated by oxidation and reactivated by reduction. It is neutralized by high titred antistreptolysin O sera. Its antibody neutralizes streptolysin O. It gives a negative lecitho-vitellin reaction. None of these characteristics apply to the haemolytic α toxin.

2. All the known oxygen-labile haemolyins which are reactivated by reduction, namely those of streptococci, pneumooccci, *Cl. tetani* and *Cl. welchii*, are closely related serologically although they are not identical in antigenic structure.

3. Those bacterial haemolysins which are not reactivated by reduction are not neutralized by the antibodies of the oxygen-labile haemolysins.

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